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A THESIS

Presented to the Faculty of

The University of Texas Graduate School of Biomedical Sciences

at San Antonio

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of the Requirements

for the Degree of

MASTER OF SCIENCE

By

Jon Eugene Piche', B.S., D.D.S.

San Antonio, Texas

May 1988

INITIAL BIOCHEMICAL CHARACTERIZATION OF CELLS DERIVED FROM HUMAN PERIODONTIUM AND THEIR IN VITRO RESPONSE TO PLATELET-DERIVED GROWTH FACTOR, EPIDERMAL GROWTH FACTOR AND TRANSFORMING GROWTH FACTOR-BETA

Jon Eugene Piche'

APPROVED:

Supervising Professor

March 18, 1988
Date

APPROVED:

Sanford A. Miller, Ph.D.
Dean

INITIAL BIOCHEMICAL CHARACTERIZATION OF CELLS DERIVED FROM HUMAN PERIODONTIUM AND THEIR IN VITRO RESPONSE TO PLAETLET-DERIVED GROWTH FACTOR, EPIDERMAL GROWTH FACTOR AND TRANSFORMING GROWTH FACTOR-BETA

Jon Eugene Piche'

The University of Texas Graduate School of Biomedical Sciences at San Antonio

Supervising Professor: Dana T. Graves

Periodontal disease is characterized by a loss of connective tissue attachment to teeth. One of the goals of periodontal therapy is to regenerate the destroyed periodontal attachment apparatus. Currently, this is not a predictable procedure. The aim of this investigation was to establish cell populations from human periodontium and partially characterize these cells using specific morphologic and biochemical criteria. In addition, the response of these cells, derived from alveolar bone, gingival connective tissue and the periodontal ligament, were

examined for their response to exogenous growth factors. Cell cultures were established from human alveolar bone (BP1), gingival connective tissue (GF2), and periodontal ligament/cementum (PL4 and PL7) using explant procedures. Subconfluent cell populations were not morphologically distinguishable. However, confluent GF2 and PL7 cultures exhibited well organized cells in monolayers, while confluent BP1 and PL4 cells grew in multiple layers of randomly oriented cells. Biochemical characterization was determined by basal levels of alkaline phosphatase activity, parathyroid hormone modulation of cyclic AMP and alkaline phosphatase levels, and 1,25 dihydroxyvitamin D3 modulation of alkaline phosphatase. Such characteristics currently distinguish bone-derived cells from fibroblasts. Parathyroid hormone induced large cyclic AMP increases in BP1 and PL4 cells and essentially no increase in GF2 and PL7 cells. Only cell population BP1 exhibited significantly decreased alkaline phosphatase activity after a fifteen minute stimulation with parathyroid hormone; while PL4 cells demonstrated significantly decreased alkaline phosphatase activity following a forty-eight hour exposure. In addition, both the BP1 and PL4 populations demonstrated a significant increase in alkaline phosphatase following a forty-eight hour exposure to 1,25 dihydroxyvitamin D3. The basal level of alkaline phosphatase activity was seven-fold higher in BP1 cells, two-fold higher in PL4 cells, and identical in PL7 cells when compared to the alkaline phosphatase activity in GF2 cells. The cell populations were subsequently examined for growth characteristics in response to exogenous growth factors. Normal human bone-derived cells proliferated in basal medium supplemented with platelet-poor plasma. The rate of proliferation was enhanced by additional supplementation with platelet-derived growth factor, and further increased when a combination of

DEDICATION

I would like to dedicate this thesis to my wife Bobbie and my children Christine and Michael. It is often one's family who has the hardest time, sacrifices the most and is ultimately responsible for the success or failure of an endeavor such as this. I would like to thank Christine and Michael for helping to show me where my priorities are and what is truly important. I would like to thank my wife, Bobbie, for the love and support she has provided to during the last three years when my time was often needed elsewhere. Her patience and love during the long hours involved in this project has helped me more than she will ever know.

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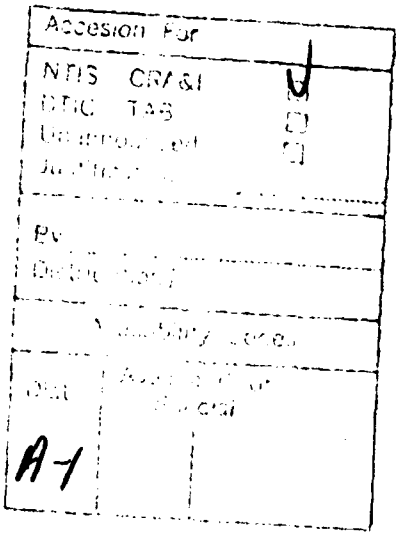
platelet-derived growth factor, transforming growth factor-beta, and epidermal growth factor was added. In contrast, normal human fibroblasts did not proliferate in basal medium supplemented with platelet-poor plasma and the addition of platelet-derived growth factor alone stimulated fibroblast proliferation to the same extent as ten-percent fetal bovine serum. Supplementation with other growth factors did not further enhance the response to platelet-derived growth factor. In addition, the PL4 cell population responded in a manner simular to the bone-derived cells and the PL7 cell population showed a similar response to the fibroblasts with regard to proliferation in platelet-poor plasma and platelet-derived growth factor. Based on this partial characterization of the cells, cell population BP1 responds in a manner consistent with previous descriptions for bone-derived cells, GF2 has a fibroblast phenotype, and PL7 has a fibroblast phenotype. Cell population PL4 does not fit precisely into either of these categories and represents an initial description of a cell population derived from the periodontal ligament/cementum which exhibits a non-fibroblast phenotype. These results also emphasize the differences in proliferative responses between human bone-derived cells and human fibroblasts, and indicate that the factors responsible for osseous regeneration in vivo may differ from those factors which regulate repair of soft tissue wounds.



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INTRODUCTION

Soft tissue wound healing and osseous regeneration following bone damage or fracture are thought to involve a similar sequence of cellular events. These include chemotaxis, proliferation of cells of mesenchymal origin at the site of injury and production of an extra-cellular matrix (Grotendorst et al., 1984). This cascade of events is largely controlled by locally generated factors that regulate the processes leading to repair or regeneration of damaged tissue. Three growth factors that are present in the early wound healing response are platelet-derived growth factor (PDGF) (Antoniades et al., 1979; Heldin et al., 1979; Antoniades, 1981; Deuel et al., 1981; Raines and Ross, 1982), transforming growth factor-B (TGF-B) (Childs et al., 1982; Assoian et al., 1983) and an epidermal growth factor (EGF)-like protein (Oka and Orth, 1983; Assoian et al., 1984). Platelets sequester these mitogens under normal circumstances and then release them upon degranulation, providing an ideal system for selectively delivering growth factors to injured tissue (Kaplan et al., 1979b). This is supported by evidence that PDGF, EGF and TGF-B stimulate soft tissue wound healing *in vivo* (Sporn et al., 1983; Grotendorst, 1984; Buckley et al., 1985; Roberts et al., 1986). Therefore, the *in vitro* response of bone cells to PDGF, EGF, and TGF-B, which are released at sites of bone injury, ought to provide insight into which factors might locally regulate osseous repair.

Recent techniques have been devised for the culture of bone cells derived from human bone explants. Beresford and his colleagues have described a technique where adult human cancellous bone fragments are cultured *in vitro* for 3-4 weeks, allowing bone cells to migrate from the

bone fragments onto a tissue culture surface (Beresford et al., 1983a; Beresford et al., 1983b; Beresford et al., 1984). Isolating cells by this technique has facilitated in vitro investigation into the cellular control mechanisms of bone-derived cells. These cells respond to PTH with an increase in cyclic AMP content (Luben et al., 1976; Auf'mkolk et al., 1985), and have high basal alkaline phosphatase activity, which is increased on exposure to 1,25-dihydroxyvitamin D3 (Beresford et al., 1986) and decreased by PTH (Wergedal and Baylink, 1984, Auf'mkolk et al., 1985; Robey and Termine, 1985). These characteristics distinguish these bone-derived cells from skin fibroblasts. Since bone cells respond differently than fibroblasts to hormonal control, they may also respond differently to locally generated paracrine factors. While the growth factor response of fibroblasts in vitro has helped identify factors which mediate soft tissue wound healing in vivo, less is known about the paracrine factors which stimulate bone cells. In order to further understand events which occur in osseous wound healing it would be helpful to identify paracrine factors that induce proliferation of bone cells. Thus, normal human bone-derived cells, isolated by the technique of Beresford and colleagues, provide a suitable population to examine the influence of paracrine factors, since they represent a population of bone cells at various stages in the osteoblast lineage that are thought to participate in osseous wound healing.

One area where osseous wound healing or regeneration is felt to play an important role is in regeneration of alveolar bone which has been lost due to periodontal disease. Periodontitis can be characterized by the loss of connective tissue attachment to teeth. The goal of periodontal therapy

is to halt this loss and ultimately regenerate the destroyed periodontal attachment apparatus. Numerous studies have shown that given the proper environment the periodontium has the capacity to regenerate a functional new attachment through the coordinated activity of osteoblasts, cementoblasts and fibroblasts (Melcher, 1970; Melcher, 1976; McCullough and Melcher, 1983; Klinge et al., 1985, Isidor et al., 1986). Based on recent research into regeneration of the periodontal attachment apparatus it is the coronal growth of the periodontal ligament which is felt to be responsible for regeneration of the periodontium (Boyko et al., 1981; Nyman et al., 1982; Gottlow et al., 1984; Isidor et al., 1986). Unfortunately, despite their importance in regeneration of the periodontium, little is known about periodontal ligament cells, particularly those found in the apical regions of the osseous defect. More information is known about osteoblasts from long bones and calvaria (Peck et al., 1964; Peck et al., 1973; Luben et al., 1976; Williams et al., 1980; Vasiliev, 1981; Nijweide et al., 1982; Wergedal and Baylink, 1984, Auf'mkolk et al., 1985; Robey and Termine, 1985) or fibroblasts from various other sources (Kulonen and Pikkarainen, 1973; Williams et al., 1980; Ham, 1984; Auf'mkolk et al., 1985). Although recent techniques have been devised for the culture of bone cells and fibroblasts from human tissue explants which has allowed <u>in vitro</u> investigation into the cellular control mechanisms of these cells, cell populations from the periodontal ligament have not yet been investigated in this manner. Therefore, a similar investigative approach needs to be taken to examine the cell types present in the periodontal ligament.

Previous efforts to characterize cells from the periodontal ligament have concentrated almost exclusively on morphologic cell characteristics (Arnold et al., 1972; Marmary et al., 1976; Ragnarsson et al., 1986). However, several cell populations are present in the periodontal ligament, including fibroblasts, osteoblasts, and cementoblasts, with all of these cells potentially contributing to periodontal regeneration (Melcher, 1976). It is therefore important to first isolate and identify cell populations derived from the human periodontal ligament. Following a similar line of investigation, these populations can then be utilized to investigate the influence of paracrine factors which are present in the periodontal wound healing environment. The results obtained for periodontal ligament cells can then be related to more extensive results which have been obtained for bone cells and fibroblasts.

II. LITERATURE REVIEW

A. Periodontal Regeneration

Periodontal disease is characterized by a loss of connective tissue attachment to teeth. Two goals of periodontal therapy therefore are to halt the attachment loss and to regenerate the destroyed periodontal attachment apparatus. The regeneration of a new periodontal attachment has been defined by Kalkwarf (1974) as the reunion of connective tissue with a root surface which has been pathologically exposed and is felt to involve the co-ordinated activity of osteoblasts, cementoblasts and fibroblasts (Melcher, 1970; Melcher, 1976; McCullough and Melcher, 1983; Klinge et al., 1985; Isidor et al., 1986). Unfortunately, the ability to selectively control these cell types is not yet a predictable clinical event. In most clinical situations it has been found that flap closure is followed by the formation of a long junctional epithelial attachment between the root surface and the soft tissue flap with epithelial cells consistently located at or close to their presurgical level (Nyman et al., 1983). In other words, repair rather than regeneration is the major physiologic response to periodontal therapy.

Fortunately, this does not mean that periodontal regeneration is not possible. A variety of therapeutic approaches have been utilized in an attempt to regenerate the lost periodontal attachment apparatus. Prichard has reported moderate success using an interdental denudation procedure which involves the excision of all soft tissue, specifically epithelium, in the interproximal region, leaving the interalveolar bone exposed (Prichard, 1957; Prichard, 1977). This technique has recently been reported by Becker

and co-workers with a similar level of success (Becker et al., 1986a; Becker et al., 1986b). Similarly, autogenous soft tissue grafts have been used to achieve regeneration in 70% of three-walled defects and 40% of two-walled defects studied. (Ellegaard & Loe, 1971; Ellegaard et al., 1974) Impressive results were achieved by Rosling an co-workers in 124 angular bony defects treated with open flap debridement, a two week maintenance schedule and scrupulous oral hygiene by the patient. After two years all of the initial defects, regardless of their initial classification, were eliminated through bone fill and some crestal bone resorption (Rosling et al, 1976). These results were essentially duplicated by Polson and Heijl (1978). Unfortunately, other investigators have not been able to duplicate these results (Caton et al., 1980; Froum et al., 1983; Isidor et al., 1985a; Renvert et al., 1985b) showing a much more limited regenerative potential following open flap debridement.

Osseous grafting is a technique which has been widely investigated with numerous authors reporting success using a variety of materials. It is generally felt that autogenous iliac marrow grafts provide the most predictable method of osseous regeneration (Schallhorn, 1977) though other autogenous grafting materials and a variety of allograft materials have been used (Schallhorn et al., 1970; Dragoo and Sullivan, 1973; Sanders et al., 1983). In a direct comparison between osseous grafting and open flap curettage, Froum, et al. (1976) found an average osseous fill of 70.6% in the grafted sites compared to 21.8% fill in the open flap curettage sites.

One of the problems with many of these studies is that they rely on clinical indicators to determine success. Periodontal probing, radiographic analysis and surgical re-entry have all been used to

demonstrate success. Unfortunately, none of these techniques can show whether a new attachment apparatus has regenerated, since this can only be accomplished through a histologic evaluation of the surgical site (Gara and Adams, 1981, Nyman et al. 1983). When histologic evaluation has been obtained following the placement of osseous grafts an apical migration of junctional epithelium has often been noted (Listgarten and Rosenberg, 1979; Moskow et al., 1979). Caton and co-workers (1980) used a monkey model to histologically evaluate periodontal regeneration following flap curettage, flap curettage and citric acid treatment, flap curettage and placement of beta tricalcium phosphate synthetic graft material, or root planing and soft tissue curettage alone. Not only did the authors find no significant differences between the four treatment modalities, they also found junctional epithelium at or close to the apical extent of the osseous defects. They did note however, that a minimal amount (<.03mm) of new cementum with inserting fibers was present in several specimens. It was concluded that before new connective tissue attachment can be proven, fiber insertion into cementum must occur in areas previously exposed to plaque inflammation or lined with pocket epithelium. This must be accomplished with the exclusion of the junctional epithelium.

Although to date attempts at regeneration have not proven to be a predictable means of achieving a new periodontal attachment, results in the literature indicate that regeneration of the attachment apparatus is possible. In a retrospective study involving 100 human block sections and extracted teeth taken from sites treated via bone and marrow autografts, allografts and nongrafting procedures, Hiatt and co-workers (1978) concluded that the potential for regeneration of a functional attachment

apparatus including new cementum, bone and a functionally oriented periodontal ligament was possible. Although regeneration did not occur in all sites, and it did not result in complete fill of the osseous defects, the results were significant in that new cementum formation was found in 66 of 79 grafted sites, new bone formation was found in 33 of 39 grafted sites, and after 6-9 months most of the periodontal ligament fibers were found to be perpendicular to the tooth and inserted in new cementum and bone. This has been confirmed by others (Cole et al., 1980; Bowers et al., 1983). These results indicate that periodontal regeneration is possible although it is often limited and confined to base of the defects. More recent studies also using histologic criteria have confirmed these results and have demonstrated the biologic possibility of regenerating a new attachment apparatus following the use of a wide variety of therapeutic modalities including open flap curettage, osseous grafts, citric acid, citric acid and fibronectin, and root submergence (Froum et al., 1983; Stahl et al., 1983, Caffesse et al., 1985; Isidor et al., 1985b; Karring et al., 1985). To date it is the opinion of most investigators that periodontal regeneration is possible, however, using the above techniques regeneration occurs primarily at the apical extent of the osseous defect and is not, as yet, a predictable procedure.

It is currently felt that there are two essential elements needed for predictable periodontal regeneration to occur. One is the exclusion of epithelium from the area and the other is the presence of cell types capable of forming the necessary hard and soft tissue structures. In studies where roots have been submerged under soft tissue flaps to completely exclude the epithelium, periodontal regeneration is a relatively

predictable event (Bowers et al., 1985; Isidor et al., 1985b; Karring et al., 1985). In order to determine whether epithelial exclusion alone is responsible for the regeneration of a new attachment apparatus, Nyman and his co-workers (Karring et al., 1980; Nyman et al., 1980) examined the ability of alveolar bone and gingival connective tissue to supply the cells necessary for regeneration. These authors found that non-diseased portions of the roots, in which the periodontal ligament had been maintained prior to implantation into bone, demonstrated a fiber reunion between the root and the surrounding tissue with a periodontal ligament reestablished between the tooth and the alveolar bone, with new cementum formation seen along the root surface. In contrast, root planed (diseased) portions of the root showed no signs of repair or regeneration with the area of the root adjacent to bone demonstrating ankylosis and the area adjacent to connective tissue demonstrating resorption. It was concluded that cells originating from alveolar bone and gingival connective tissue do not appear to be capable of initiating attachment formation to teeth which have previously been pathologically exposed. (Karring et al., 1980; Nyman et al., 1980; Karring et al., 1984). Indeed, it appears that these cell types are more likely to initiate ankylosis or resorption of the root surface if they are not excluded from the area (Loe & Waerhaug 1961; Line et al., 1974; Karring et al., 1980; Lopez and Belvederessi, 1983; Isdor et al., 1986).

In 1976, Melcher (Melcher, 1976) postulated that the cells which repopulate the root surface will determine the type of attachment which subsequently forms. He felt that the periodontal ligament was of prime importance in that it provides continuity between the alveolar bone and

cementum and also because it is felt to contain cells that can synthesize and remodel the three connective tissues of the periodontium, namely alveolar bone, cementum and gingival connective tissue. Subsequently, using light microscopy these three cell types have all been identified in the periodontal ligament (Melcher 1980; Berkovitz and Shore, 1982), in addition to possible progenitor cells, which are also felt to be necessary to regenerate the periodontal attachment (Gould, 1983; McCulloch and Melcher, 1983; McCulloch, 1985).

Recent evidence indicates that it is the coronal growth of the peridontal ligament following therapy which is primarily responsible for the formation of a functional attachment apparatus (Boyko et al. 1981, Nyman et al. 1982, Isidor et al. 1986). In a study using beagle dogs, Boyko, Melcher and Brunette (1981) demonstrated that cultured periodontal ligament cells were able to produce areas of new periodontal ligament in vivo when reimplanted onto demineralized roots, compared to control teeth reimplanted with gingival connective tissue cells. The newly formed periodontal ligament was identified on the basis of fiber orientation, the presence of Sharpey's fibers between the bone and the root, and the location of cells in positions normally occupied by osteoblasts and cementoblasts. More recently, Isidor and co-workers (1986), using a monkey model, demonstrated that submerged teeth with an elastic ligature placed tightly around each tooth, to preclude the coronal migration of the periodontal ligament, had a new attachment which was apical to the level of the ligature in 7 of 8 teeth, compared to loosely ligated teeth which had new attachment inserting into newly formed cementum above the ligature in all 10 of the teeth. In both groups the roots exhibited a significant

amount of resorption coronal to the newly formed cementum. These findings were felt to support the concept that cells migrating from the periodontal ligament have the potential to form cementum and a new connective tissue attachment and that repopulation of the root surface by cells from the periodontal ligament is a prerequisite for new attachment formation. Unfortunately, in the absence of some form of a labeled cell marker, this animal model system does not prove that the cells which formed the new attachment to the tooth were actually derived only from the periodontal ligament.

To date the best method for achieving periodontal regeneration involves the mechanical exclusion of gingival connective tissue as well as junctional epithelium from the involved site through the use of a millipore filter which is placed between the tooth surface and the gingival connective tissue. This technique has been widely reported by Nyman, Gottlow and co-workers in both animals (Nyman et al., 1982a; Gottlow et al., 1984; Magnusson et al., 1985) and humans (Nyman et al. 1982b; Gottlow et al., 1986) with the results recently confirmed by other independent investigators (Aukhil et al., 1983; Caton et al., 1987; Aukhil et al., 1987). In a recently published report, Gottlow and co-workers (1986) present histologic evidence of significant new attachment formation in humans in both vertical osseous defects and furcation defects. Though these results are encouraging, it was been noted by the authors (Gottlow et al., 1986) that new attachment and cementum formation occurs almost exclusively in areas of angular bony defects. It is felt that using current techniques, the periodontal ligament cells have a limited coronal

migratory potential which inhibits coronal gain of attachment in horizontal defects.

B. Wound Healing

Soft tissue wound healing and osseous regeneration following bone damage or fracture are felt to involve a similar sequence of cellular events. Since periodontal disease also involves the loss of soft tissue structures, such as the periodontal ligament and its connective tissue attachment to the tooth, and hard tissue structures, such as alveolar bone and cementum, regeneration or repair of the periodontium would also involve the same sequence of basic cellular events. These events include cell chemotaxis, proliferation of cells at the site of damage, and production of an extra-cellular matrix (Howes and Hoopes, 1977; Shoshan, 1981; Grotendorst et al., 1984). As such, one can look at events which occur during wound healing and apply this information to the periodontium. After injury, the damaged tissue is sequentially invaded by inflammatory cells, connective tissue cells, and endothelial cells. It is felt that each cell type (ie. blood cells, injured cells, inflammatory cells, etc.) which arrives on the scene produces polypeptide factors which in turn recruit additional cell types, stimulate cell proliferation and production of an extracellular matrix. It is these polypeptide factors which are largely responsible for controlling the type and the amount of cells responding at the injured site (Grotendorst et al., 1984; Grotendorst. 1984; Sporn and Roberts, 1986).

Chemotaxis is one of the initial events in wound healing and can be defined as a directed movement of cells along a chemical gradient

(Snyderman, 1986). Cell chemotaxis is controlled by factors which are present in the wound environment. The chemotactic factors are specific for a particular cell type, which is the way the types of cells and the sequence of arrival is regulated. For a cell to be activated it must first bind to the chemotactic molecule (factor) with each cell type possessing a unique set of receptors governing the specificity of the response (Snyderman et al., 1970; Postlethwaite et al., 1976; Postlethwaite et al., 1981; Snyderman, 1986). One of the better described binding interractions occurs between fibroblasts and platelet-derived growth factor and can serve as a model for understanding this important phenomenon. PDGF directly binds to target cells at high affinity specific receptors which are located on the cell surface (Heldin et al., 1981; Bowen-Pope and Ross, 1982; Heldin et al., 1982; Williams et al., 1984). The target cells (ie. fibroblasts) contain a limited number of receptors which have a high affinity for PDGF as evidenced by a dissociation constant in the range of 10^{-9} to 10^{-11} (Heldin et al., 1981; Bowen-Pope and Ross, 1982). Binding is felt to occur between the PDGF molecule and a large molecular weight (164,000 daltons) surface protein which possesses protein kinase activity and consists of a single polypeptide chain (Glenn et al., 1982; Williams et al., 1984). The actual binding is temperature dependent occurring more rapidly at $37^{o}C$ than at $5^{o}C$. The bond quickly becomes nondissociable and the PDGF is internalized within 5-10 minutes in small endocytotic vessicles where it is transported to lysosomes. As the PDGF is internalized there is a decrease in the number of surface receptors resulting in a downregulation of the cell to PDGF. By 60 minutes the PDGF appears in the Golgi cisternae and vesicles where it is subsequently degraded (Nilsson et al., 1983).

Currently the mechanism by which the PDGF activates the cell is unknown, though the tyrosine kinase activity of the receptor is thought to be involved (Ek and Heldin, 1982; Ek et al., 1984; Kazlauskas et al., 1986). Interestingly chemotaxis and mitogenesis are probably controlled by different binding sites on the cell membranes (Williams et al., 1983; Senior et al. 1985).

Following injury, the first cell types to arrive in response to the chemotactic signal are the blood cells, primarily the platelets (Ross, 1980; Knighton et al., 1982; Grotendorst, 1984). Platelets are known to sequester a variety of chemotactic and growth factors under normal circumstances and then release them upon degranulation at the site of injury. Therefore, platelets are thought to be an ideal system for selectively delivering these factors to sites of injury (Kaplan et al., 1979b). This is followed by the accumulation of phagocytic cells in the wound. These cells are probably responding to chemotactic factors which are released by the damaged tissue or by the platelets. Tzeng and her colleagues demonstrated that platelet-derived growth factor is able to activate human polymorphonuclear leukocytes, as measured by chemotaxis and subsequent neutrophil degranulation, at concentrations below those found in the serum (Tzeng et al., 1984). Chemotaxis to PDGF has also been demonstrated for monocytes/macrophages (Deuel et al. 1982). This is important since neutrophils are the first phagocytic cell to arrive at the wound site, followed within 24 hours by the macrophages. These cells in turn contribute other chemotactic factors which, by the third or fourth day, attract endothelial cells and connective tissue cells such as fibroblasts and smooth muscle cells (Glaser et al., 1980; Postlethwaite et

al., 1979; Banda et al., 1982; Grotendorst et al., 1984). The connective tissue cells have been shown to respond to a variety of chemattractive factors such as collagen and collagen fragments (Postlethwaite et al., 1978), lymphokines (Postlethwaite et al. 1976), fibronectin (Gauss-Muller et al., 1980) and platelet-derived growth factor (Seppa et al., 1982). Seppa and co-workers (1982) were able to show that platelet-derived growth factor strongly stimulated fibroblast chemotaxis and that PDGF was the major chemotactic substance released by platelets. Other growth factors such as epidermal growth factor (EGF), fibroblast growth factor(FGF) and insulin were not found to be chemotactic, and currently it is felt that PDGF is an important chemoattractant during this initial phase in wound healing.

Following migration of the connective tissue cells into the injured area they begin to undergo mitogenesis with an increase in cell number. It is during this proliferative phase of wound healing that there is an increase in the number of the cells which will ultimately replace the lost or damaged tissues. Cell types which proliferate at the site of injury include endothelial cells, epithelial cells, fibroblasts, osteoblasts, and in the periodontal wound healing environment, cementoblasts. It is felt that polypeptide growth factors are primarily responsible for this proliferative response. Three growth factors that are released from platelets and are present in the early wound healing response are platelet-derived growth factor (PDGF) (Antoniades et al., 1979; Heldin et al., 1979; Deuel et al., 1981; Raines and Ross, 1982), transforming growth factor-B (TGF-B) (Childs et al., 1982; Assoian et al., 1983) and an epidermal growth factor-like protein (Oka and Orth, 1983; Assoian et al.,

1984). In addition, blood monocytes release mitogens such as PDGF (Shimokado et al., 1985; Martinet et al., 1986), interleukin I (IL-I) (Luger et al., 1983) and fibroblast growth factor (FGF) (Baird et al., 1985); while endothelial cells produce a platelet-derived growth factor-like protein (DiCorleto and Bowen-Pope, 1983). These polypeptide growth factors are called paracrine factors because they are released locally and exert their function in a limited area, as opposed to autocrine factors which are secreted by cell types residing in the immediate area (Sporn and Toduro, 1980) and endocrine factors which are systemic. Since the growth response occurs in a limited area, the factors that control wound healing necessarily act in a limited area.

Much of the investigation into the cellular mechanisms of wound healing have utilized tissue culture systems. It is known that when cells in culture are deprived of essential nutrients and growth factors they cease to divide and come to rest in the G_1 phase of the cell cycle. These cells are termed quiescent and enter a state which is termed G_0 where by they are viable and functioning but have exited the proliferative cycle. This G_0 state is felt to be analagous to the normal state of somatic cells which are not actively dividing (Martin and Stein, 1976; Moses et al., 1978; Pardee et al., 1978; Antoniades and Owen, 1982). In order for a growth factor to initiate DNA synthesis and cell division in a connective tissue cell which exists in the G_0 state it must first cause the cell to re-enter the G_1 phase with subsequent transition to the S phase of the cell cycle. This occurs in two discrete stages, termed competence and progression, which the cell must pass through in order to become committed to leave the G_0 phase. Both competence and progression factors have been

shown to be present in plasma (Temin et al., 1972). Platelet-derived growth factor is felt to be the principle competence factor contained within platelets (Pledger et al., 1977), which allows the cell to leave the G_0 phase, and serum contains additional progression factors, such as Somatomedin C (Insulin-like growth factor) (Stiles et al., 1979), which allows the cell to progress through the S phase of the cell cycle. Competence and progression factors have also been shown to be produced by macrophages (Glenn and Ross, 1981). Both Shimokado and co-workers (1985), and Martinet and co-workers (1986) have isolated a chemical mediator from activated monocytes/macrophages which is capable of stimulating chemotaxis and cellular proliferation of a variety of mesenchymal cells, including smooth muscle cells, fibroblasts, 3T3 cells, and vascular endothelial cells. On closer examination this factor which is secreted by the monocytes/macrophages has been shown to be essentially identical to platelet-derived growth factor in terms of its biochemical structure and range of activity. As such, the monocyte/macrophage can be viewed as another potential source of competence factors in the wound healing environment. Therefore, it is currently felt that in the wound healing environment connective tissue cells such as fibroblasts, smooth muscle cells and possibly osteoblasts, must become activated through the action of competence and progression factors found in the blood before cell proliferation and subsequent tissue regeneration or repair can occur.

A final event in wound healing is the formation of an extracellular matrix by the cells which are present at the wound. The major structural component present is collagen which, in addition to giving strength and support to the tissues, also functions in cell adhesion and migration.

There are multiple forms of collagen produced by the different cells of which five forms are most common. For example, fibroblasts and osteoblasts produce primarily type I collagen and a small amount of type III collagen. Large glycoproteins are also present which contain specific binding sites for the collagens, proteoglycans and other cell types. Again, there are multiple types of glycoproteins, the best characterized of which is fibronectin (Kleinman et al., 1981) which is responsible for attaching fibroblasts and smooth muscle cells to type I and type III collagen. Fibronectin is also a chemoattractant for multiple cell types, such as fibroblasts and macrophages (Postlethwaite et al., 1981; Grotendorst et al., 1984). An additional element of the extracellular matrix are the proteoglycans (ie. chondroitin sulfate, heparin sulfate, etc.). These components serve a variety of important functions such as hydration and filtration (Grotendorst et al. 1984). Therefore, it is through the production and maturation of the extracellular matrix that tissue repair and regeneration ultimately occurs.

C. Cell Types Which Are Important In Periodontal Regeneration

There are basically three differentiated cell types which are involved in periodontal regeneration, namely osteoblasts, fibroblasts, and cementoblasts (Melcher, 1976). While much has been written concerning fibroblasts, and considerable information is becoming available regarding the mechanisms which control bone regeneration, little is known regarding the proliferative capabilities of cementoblasts.

1. Bone-Derived Cells: Isolation and Characterization. Bone formation is a complex process which is regulated by hormones, systemic

growth factors, and local growth factors. Although the in vitro growth factor requirements of fibroblasts and other soft tissue cells have been systematically examined the growth factor requirements of bone and bone-derived cells are unclear. This is because bone formation and resorption is a complex process which is difficult to study using currently available in vitro and in vivo systems.

There are currently three well established in vitro models for studying bone cells: 1. cultures of intact fetal rat calvariae, 2. cultures of normal calvarial cells enriched in osteoblasts, and 3. cultures of immortalized, osteoblast-like cell lines. There are advantages and disadvantages of each model system. Although calvarial cultures preserve normal tissue anatomy, they contain a mixed-cell population making interpretation of results difficult regarding a specific cell population (ie. osteoblasts). Similar difficulties are present in osteoblast-enriched calvarial cell cultures. Immortalized cell populations have the problem in that you are dealing with a cell line which may not behave in the same manner as a normal cell population (Canalis, 1985). More recently, techniques have been devised for the culture of bone cells derived from normal human explants (Beresford et al., 1983a; Beresford et al., 1984; Auf'mkolk et al., 1985). This has allowed further investigation into many of the factors controlling bone regeneration and resorption.

2. Bone-Derived Cells: Response to Hormonal Stimulation. One of the most thoroughly investigated aspects of in vitro bone cell cultures is the hormonal regulation of bone-derived cells. Bone-derived cells are thought to represent several stages in the osteoblast lineage and it is through their response to hormonal stimulation that cells with an

osteoblast-like phenotype are differentiated from other non-osteoblast cells. One of the hormones which has been widely utilized to characterize bone cell populations is parathyroid hormone. PTH is known to induce bone resorption both in vivo and in vitro, compared to calcitonin which inhibits bone resorption (Wong and Cohen, 1974). Peck and co-workers, using a collagenase digestion protocol to obtain a mixed population of cells from fetal rat calvariae, were among the first to investigate the response of bone-derived cells to hormonal stimulation. They found that a high concentration of purifed bovine PTH (100 ng/ml) in the presence of theophylline, inhibited c-AMP phosphodiesterase, causing a 14-fold increase in the level of cyclic-AMP within 30 seconds. At physiologic concentrations (1 ng/ml) the authors found a decreased, but still statistically significant, 25% increase in cyclic-AMP levels (Peck et al., 1964; Peck et al., 1973). Smith and Johnston (1975) found a similar increase in cyclic-AMP following exposure to PTH and calcitonin. These findings were extended by Wong and her colleagues, who utilized the same cell culture system while performing multiple, sequential, collagenase digestions on the calvaria to obtain multiple cell populations. These populations were found to differ significantly in their response to PTH and calcitonin. The cells released early in the digestion were found to show a two-fold increase in cyclic-AMP folowing both calcitonin and PTH stimulation, while the later cell populations demonstrated a significantly greater response to PTH and no response to calcitonin. In addition, the early digestion cells had higher levels of acid phosphatase and hyaluronate synthesis which were increased 100-200% following exposure to PTH. In contrast, the late digestion cells had higher basal levels of alkaline

phosphatase, citrate decarboxylation, and prolyl hydroxylase, which decreased 75-90% following exposure to PTH. The authors concluded that the cells obtained early in the digestion process were enriched with osteoclasts while those obtained late in the digestion process were enriched with osteoblasts (Wong and Cohn, 1974; Wong and Cohn, 1975; Luben et al., 1976). This is significant in that these investigators were able to clearly show that multiple cell populations were obtained from bone organ cultures and that these populations could be differentiated based on their response to hormones such as PTH and calcitonin.

Since these initial studies were performed numerous other investigators have utilized biochemical tests to investigate bone-derived cells in tissue culture and gain a better understanding into the hormonal controls of osteoblast-like cells. The ability of PTH to decrease alkaline phosphatase has been confirmed in bone organ cultures (Thomas and Ramp, 1979), and also demonstrated in an osteoblast-like clonal cell line derived from rat osteosarcoma (ROS 17/2) (Majeska and Rodan, 1982). The effects of 1,25 dihydroxyvitamin D3, a steroid hormone which is involved in tissue mineralization through the uptake of calcium, have been widely investigated in bone organ cuture and clonal osteosarcoma cell lines. Specifically, 1,25 dihydroxyvitamin D3 and its analogs have been shown to decrease collagen synthesis, while increasing alkaline phosphatase and osteocalcin levels (Manolagas et al., 1981; Brand et al., 1982; Bringhurst and Potts, 1982; Canalis, 1983; Chen et al., 1986). When the collagen type was analysed it was found that bone-derived cells produced predominantly type I collagen (94%) with small amounts of type III and type V collagen (Whitson et al., 1984). An additional marker for osteoblast-like cells which has

recently been investigated, is the ability of these cells to form a calcified bone matrix under certain tissue culture conditions. This has been reported by numerous laboratories and is felt to be a specific marker for osteoblast-like cells since neither osteoclasts nor fibroblasts are felt to form calcified matrices (Williams et al., 1980; Nijweide et al., 1982; Ecarot-Charrier et al., 1983; Sudo et al., 1983; Whitson et al., 1984).

Recently, techniques have been devised for the culture of bone cells derived from human bone explants. Previous investigations of bone-derived cells primarily utilized either embryonic rodent calvaria subjected to sequential collagenase digestion, or clonal cell lines obtained from osteosarcoma cells. The ability to extrapolate these results to normal human bone-derived cells is unknown. In addition to obtaining bone-derived cell populations using the previously reported collagenase digestion protocol (Wergedal and Baylink, 1984; Robey and Termine, 1985), several laboratories have developed a system where normal human bone is utilized as a donor tissue and cells are allowed to migrate from the bone and form tissue culture populations (Beresford et al., 1983a; Beresford et al., 1983b; Auf'mkolk et al., 1985). Under identical assay conditions, no difference was found by Auf'mkolk and colleagues in the cells obtained from either culture method to endogenously applied hormones. These cell populations have been found to respond to endogenously applied hormones in the following manner. In a series of experiments Beresford and his co-workers found that normal human bone-derived cells produce a variety of collagenous and non-collagenous tissue proteins which are predictably modulated by PTH and 1,25 dihydroxyvitamin D3. One of the non-collagenous

proteins investigated was osteocalcin which is the most abundant non-collagenous protein in bone and is felt to play an undefined role in regulating bone mineralization. Osteocalcin, which is present in tissue culture populations of bone cells, was found to be present at increased levels following exposure to 1,25 dihydroxyvitamin D3, and at decreased levels following exposure to parathyroid hormone, interleukin-1, and prednisolone (Beresford et al., 1984a, Beresford et al., 1984b; Skjodt et al., 1985). In addition, 1,25 dihydroxyvitamin D3 was found to increase alkaline phosphatase and type I collagen production, while decreasing prostaglandin E and total protein production in the cell cultures (MacDonald et al., 1984; Beresford et al., 1986). Conversely, PTH was found to increase prostaglandin E production (MacDonald et al., 1984). In a similar culture system Aufm'kolk and colleagues (1985) utilized multiple explants of normal human bone cells and confirmed that the cells produce osteocalcin, which was increased upon stimulation with PTH and 1,25 dihydroxyvitamin D3; had high basal levels of alkaline phosphatase, which were decreased upon exposure to these hormones; and showed increased cyclic-AMP production following exposure to PTH. Collagen type was assayed and found to be similar to previous descriptions with >95% type I collagen and the remainder type III collagen, which is consistent with a population consisting of osteoblast-like cells. The response of the cell populations was found to decrease with increased cell passage, which others have also reported (Wong and Cohn, 1974; Luben et al., 1976). In contrast to the bone-derived cells, normal human fibroblasts either did not respond or showed a very minimal response to PTH and 1,25 dihydroxyvitamin D3 exposure, did not produce osteocalcin, and had low basal levels of alkaline

phosphatase. In addition, a much higher proportion of type III collagen was produced by the fibroblast cultures (Auf' mkolk et al., 1985). Similar results were obtained by Robey and Termine (1985) using human bone cells obtained through collagenase digestion. The results from these investigations are significant in that they utilize normal human bone cells and show that these cells respond predictably to certain biochemical markers, which can then be used to differentiate these cell populations from other cell populations such as osteoclasts and fibroblasts. In addition, these results duplicate and confirm the results obtained in earlier investigations which utilized clonally derived cell lines from osteosarcoma cells and bone organ cultures from rodent calvaria. Currently it is necessary to demonstrate a response to hormonal modulation prior to assigning an osteoblast-like phenotype to a bone-derived cell population.

3. Bone-Derived Cells: Response to Growth Factors Although the hormonal regulation of bone-derived cells has been thoroughly investigated, the response to growth factors is less well understood. Despite these limitations, a number of growth factors have been studied in bone organ cultures or osteoblast-like cultures. Platelet derived growth factor has been found to stimulate DNA synthesis and noncollagenous protein synthesis in cultured rat calvaria, and collagen and bone resorption in neonatal mice calvaria (Canalis, 1981; Tashijan et al., 1982). Human osteosarcoma cells bind labelled PDGF and demonstrate increased membrane phosphorylation, amino acid transport, DNA synthesis, and proliferation in response to PDGF (Graves et al., 1984). More recently, Hanks and colleagues demonstrated in rat calvarial cultures tha PDGF stimulates early protein synthesis allowing quiescent cells to become competent to progress through the G_1

phase of cell division. These cells then required addition of platelet-poor plasma or EGF and SM-C/IGF to progress through to the S phase and undergo cell division (Hanks et al., 1986). This indicates a regulatory role for PDGF in bone similar to that seen with fibroblasts. Canalis and Raisz have shown that nanomolar concentrations of EGF stimulate DNA synthesis, but inhibit both collagen synthesis and alkaline phosphatase activity in cultured rat calveria (Canalis and Raisz, 1979). Kumegawa and co-workers demonstrated that epidermal growth factor induces a dose-related decrease in collagen synthesis and alkaline phosphatase activity, and an increase in total protein synthesis in the MC3T3-E1 osteoblast-like cell line (Kumegawa et al., 1983). Similarly, Ng and colleagues found that EGF stimulated an increase in DNA synthesis and cell proliferation, following a 6-8 hour lag period, in a clonal line of rat osteogenic sarcoma cells (UMR 106) and in osteoblast-rich, newborn rat calvarial cells (Ng et al., 1983a). Although SM-C/IGF has been shown to stimulate DNA, RNA and glycogen synthesis in cultured calvarial cells, this increase is small compared to the response seen in serum, suggesting that other growth factors or hormone are needed for maximal response (Canalis, 1980; Schmid et al., 1983). SM-C/IGF have also been found to stimulate increased alkaline phosphatase activity and type I collagen synthesis (Schmid et al., 1984). Recently the effects of TGF-beta on bone organ cultures have been described. While DNA synthesis has been shown to increase in fetal rat calvaria and osteoblast-enriched fetal rat bone cultures, TGF-beta has been found to have no affect on collagen synthesis, non-collagenous protein synthesis, and alkaline phosphatase activity (Centrella et al., 1986; Centrella et al., 1987). Recently, Robey and co-workers (1987)

demonstrated that fetal bovine bone cells produced TGF-beta and the TGF-beta then caused an increase in cell growth but not an increase in collagen synthesis. In addition, TGF-beta has been shown to increase prostaglandin production with a resultant increase in bone resorption (Tashjian et al., 1985). Since TGF-beta is involved in both bone formation and bone resorption, it may act as an independent effector if the two processes are independent, or a local coupling factor if the two processes are functionally related (Centrella and Canalis, 1985). Therefore, significant information regarding the role of locally present growth factors in the regulation of bone resorption and bone formation is known. Unfortunately, the applicability of this information to normal human bone cells is currently unknown. To date, the addition of exogenously applied growth factors to normal human bone-derived cells has not been reported in the literature.

4. Fibroblasts. The fibroblast is another cell type which is important in wound healing or regeneration. As previously discussed, these cells are intimately involved in the initial events in wound healing. As the predominant cell type involved in soft tissue wound healing, fibroblasts play a major role in the production of chemotactic factors, growth factors, and the extracellular matrix (Shoshan, 1981).

To date, the in vitro characteristics of fibroblasts have been well studied (Kulonen and Pikkarainen, 1972, Ham, 1984), with the response of these cells to hormone and growth factor modulation having been extensively investigated. Characteristically, fibroblasts are utilized as a comparison for other cell types when examining the response to certain hormones. For example, Auf'mkolk and co-workers (1985) compared human skin fibroblast and

human bone cell response to hormonal modulation, finding that while bone cells responded significantly to parathyroid hormone and 1,25 dihydroxyvitamin D3, fibroblasts showed little or no response. In this way the investigators were able to differentiate the two cell types based on certain biochemical characteristics. With regard to the investigation of growth factors, fibroblasts are the cell type commonly used to examine *in vitro* and *in vivo* responses to these chemical mediators. Much of the information concerning platelet-derived growth factor (Antoniades and Owen, 1984), epidermal growth factor (Carpenter and Cohen, 1979), insulin-like growth factor (Van Wyk, 1984), and transforming growth factor-beta (Sporn et al., 1986b), which are growth factors felt to be involved in wound healing, was originally obtained utilizing fibroblasts in tissue culture systems (Sporn and Roberts, 1986). Therefore, the growth factor requirements of fibroblasts have been well investigated, and fibroblasts continue to be extensively utilized.

5. *Cementoblasts*. In contrast to the information available concerning the role of fibroblasts and osteoblasts in wound healing, little information is known regarding the response of cementoblasts in a periodontal wound healing environment, specifically with regard to their modulation by growth factors. Although cementum does not undergo physiological resorption or remodeling like bone, it is continuously deposited throughout life. In addition, as previously described, cementum has the potential to regenerate, though as yet not in a predictable manner. Therefore, cementoblasts must respond in some manner to chemical factors known to preside locally in the periodontal environment. Unfortunately, how these cells respond is not yet known.

Several authors have described tissue culture systems for the in vitro study of periodontal ligament cells (Arnold and Baram, 1972; Marmary et al., 1976; Blomlof and Otteskog, 1981; Ragnarsson et al., 1985). With the exception of Marmary and his co-workers (1976) who reported that the c-AMP response to parathyroid hormone in explants obtained from the periodontal ligament was similar to that seen in a population of skin fibroblasts, the extent of cell characterization in the remaining studies involves only a morphologic description of the periodontal ligament cells as they appear in tissue culture. Recently, Rose and colleagues (1987) investigated the ultrastructural features of human periodontal ligament cells which were primary cell cultures or were continuous lines. They found that these cells contained dense accumulations of glycogen and were similar in appearance to cementoblasts. These results were similar to an earlier in vivo study of the periodontal ligament by these authors (Yamasaki et al., 1986) and as such these cells were felt to be cementoblastic. Therefore, while initial strides have been made in the isolation and identification of cementoblast populations from the periodontal ligament, as yet, no information is known regarding the modulation of cementoblasts by chemical mediators which are present in the local environment.

D. Growth Factors

1. Platelet-Derived Growth Factor (PDGF). As was previously described, the formation of collagen and its binding via glycoproteins is a late event in wound healing. It is not considered to be the regulating or rate-limiting step in the regenerative process since it is controlled by the migration and proliferation of the cells (ie. fibroblasts, smooth

muscle cells, etc.) into the wound area which is felt to regulate the rate of healing. In this way, it is the polypeptide growth factors which are primarily responsible for directing and controlling the cellular events which are responsible for wound healing. It is important to have an understanding of some of the more prevalent growth factors in order to better understand the biology of the wound healing environment. One of the principle growth factors which is felt to be involved in soft and hard tissue wound healing is platelet-derived growth factor (Sporn and Roberts, 1986a). Platelet-derived growth factor (PDGF) is a dimeric protein made up of two different polypeptides, each encoded by a different gene. PDGF has been shown to be synthesized by megakaryocyte cell lines and stored in platelets. It is also produced by activated macrophages (Antoniades et al., 1975; Antoniades et al., 1979; Heldin et al., 1979; Deuel et al., 1981; Raines and Ross, 1982; Raines and Ross, 1985). It is estimated that each platelet contains approximately 1000 molecules of PDGF (Stiles, 1983).

The platelet presents a unique means of delivering the growth factor to the site of injury. It should be remembered that mature cells are normally exposed only to plasma (ie. no platelets) or a filtrate of plasma (ie. interstitial fluid) (Ross and Vogel, 1978) and consequently will usually not contact the platelets and their contents (ie. PDGF, TGF-beta). However, upon injury platelets migrate to the site of injury, degranulate and release their contents (ie. alpha-granules) into the local environment. This presents a system for delivering PDGF only to the site of injury to assist with wound healing. Using a baboon model, Bowen-Pope and colleagues (1984) found that PDGF injected intravenously was rapidly cleared from the plasma, with a half-life of less than two minutes. This also supports the

concept that it is the local secretion and synthesis which is responsible for the activity of PDGF rather than circulating levels. More recently PDGF and PDGF-like molecules have been identified as products of macrophages, smooth muscle cells, and endothelial cells (DiCorleto and Bowen-Pope, 1983; Nilsson et al., 1985; Shimokado et al., 1985; Martinet et al., 1986; Ross et al. 1986), providing an additional means of PDGF delivery to an injured site.

Consistent with its role as the major growth factor in a wound healing environment, a variety of biological effects have been proposed for PDGF. To date, high affinity cell-surface receptors for PDGF have only been demonstrated on mesenchymal cells, such as fibroblasts, smooth muscle cells and glial cells (Ross et al., 1986). Since surface receptors are necessary for PDGF to elicit a response, it is felt that the action of PDGF is primarily confined to cells of mesenchymal origin. The physiologic effect which has been most extensively investigated is the ability of PDGF to stimulate DNA synthesis and cell division in fibroblasts (Antoniades et al., 1975; Pledger et al., 1977; Ross and Vogel, 1978; Owen et al., 1982; Antoniades and Owen, 1984), human brain glial cells (Westermark and Wasteson, 1976), and smooth muscle cells (Ross et al., 1974; Ross and Vogel, 1978). In a classic experiment, Pledger and co-workers (1977) determined that PDGF is the major competence factor in serum. In addition to establishing competence PDGF acts synergistically with progression factors (ie. insulin-like growth factor, epidermal growth factor) to stimulate DNA synthesis and cell growth (Stiles et al., 1979). The growth factor activity of serum was originally observed by Balk and co-workers (Balk, 1971; Balk et al., 1973), who reported that normal fibroblasts

proliferated well in culture media supplemented with serum compared to media supplemented only with platelet-poor plasma. It was these observations, which were later extended by several investigators (Ross et al., 1974; Kohler and Lipton, 1974) leading to the connection between serum growth factors and blood platelets and later to the isolation of PDGF from platelets. Further proof for the ability of PDGF to induce competence is its ability to modulate the production of mRNA and proteins by the affected cell. When production of these proteins is blocked, competence does not occur (Antoniades and Owen, 1984). It is through the modulation of DNA synthesis and cellular proliferation that PDGF is felt by most investigators to have its major in vivo impact on wound healing.

Another proposed physiologic role for PDGF is as a chemotactic agent for a variety of cell types in the wound healing environment. PDGF has been shown to stimulate chemotaxis of fibroblasts (Seppa et al., 1982), smooth muscle cells (Grotendorst et al., 1982), brain astrocytes (Bressler et al., 1985), and neutrophils and monocytes (Deuel et al, 1982). Other growth factors such as, FGF, EGF, NGF, and insulin have not been found to be chemotactic to fibroblasts (Seppa et al., 1982). This serves to further highlight the probable importance of PDGF as a pivotal growth factor in the body's wound healing response.

In addition to cell proliferation and chemotaxis, PDGF has been shown to modulate a variety of other cellular metabolic activities. Owen and co-workers (1982) have shown that when PDGF is added to quiescent human fibroblasts, amino acid uptake is doubled within two to three hours. The increase in amino acid uptake is related to increased protein synthesis. Consistent with its role as a wound healing factor, PDGF has also been

shown to regulate the production of extracellular matrix components. Collagen synthesis, as well as glycosaminoglycan and proteoglycan synthesis have been shown to be enhanced by PDGF (Burke and Ross, 1977; Castor et al., 1977). More recently, Narayanan and Page (1983) found that PDGF specifically stimulates type V collagen formation by gingival fibroblasts along with regulating the synthesis of type III versus type IV collagen. In contrast, it has also been observed that 8–10 hours following exposure to PDGF, collagenase secretion is stimulated (Bauer et al., 1985). This serves to point out the complex in vivo role of PDGF. It has also been shown that PDGF causes an increase in cholesterol ester uptake and synthesis in human fibroblasts by increasing the number of LDL receptors (Chait et al., 1980; Witte and Cornicelli, 1980; Leslie et al., 1982) along with an increase in prostacyclin (PGI_2) synthesis by endothelial and smooth muscle cells (Coughlin et al., 1980; Coughlin et al., 1981), and an increase in prostaglandin E_2 (PGE_2) synthesis (Tashjian et al., 1982; Rozengurt et al., 1983). Lipid synthesis has been shown to occur several hours prior to DNA synthesis and may play a role in membrane construction during cell proliferation (Leslie et al., 1982). Prostacyclin is a vasodilator and potent inhibitor of platelet aggregation. It is felt that the increased production of PGI_2 by PDGF is part of a negative feedback mechanism that controls platelet aggregation and vasoconstriction in the initial stages of the wound healing environment, thus preventing excess platelet accumulation and thrombus formation. Prostaglandin E_2 is a known metabolite of arachidonic acid and is felt to be involved in bone resorption (Raisz and Kream, 1983), possibly through the action of blood monocytes (Key et al., 1983). The increase in prostaglandin production has

also been shown to coincide with an increase in cAMP levels, which may signal the initiation of cell proliferation (Rozengurt et al., 1983). Other metabolic events which have been shown to occur shortly after exposure to PDGF include the rapid rearrangement of preformed actin and myosin microfilaments which has been reported in fibroblasts (Bockus and Stiles, 1984), and glial cells (Westermark et al., 1983), enhancement of glycolysis, and stimulation of polysome formation (Deuel and Huang, 1984).

Recently the in vivo effects of PDGF have begun to be investigated using an animal model system. Grotendorst and co-workers (1984) implanted stainless steel mesh chambers subcutaneously in rats. The chambers were then filled with type I collagen gel (2 mg/ml) with or without PDGF (50 ng/ml) to compare the wound healing response. Pilot studies determined that the collagen gel alone did not change the amount of DNA synthesis or collagen deposition compared to empty chambers. The authors found that the addition of PDGF resulted in a significant increase in DNA synthesis and collagen deposition in the chambers in the first days after implantation. Histologically, the tissue found in the PDGF and non-PDGF chambers was identical, indicating that the addition of PDGF promoted normal healing and not a repair or fibrosis response. The authors conclude that the wound healing response in normal animals may be increased by supplementation of the wound with growth factors such as PDGF. In contrast, Leitzel and co-workers (1985) using a hamster model found the PDGF, EGF and FGF did not increase the rate of healing when topically applied to full thickness skin wounds. The dissimilarity of the two models makes direct comparison of the data impossible; however, the rapid clearance of PDGF at a wound site may help explain the difference in results. In addition, as Leitzel and

colleagues point-out, their model may already contain a maximum amount of PDGF and therefore any additional supplementation would not be expected to increase the rate of wound healing. Recently, Lynch and co-workers (1987) investigated the effect of purified PDGF and partially purified PDGF alone and in combination with EGF and IGF-1 on wound healing in White Yorkshire pigs. They found that purified PDGF alone, EGF, IGF-1 or purified PDGF in combination caused little increase in production of extracellular connective tissue components and was of little value in accelerating normal wound repair. In contrast, partially purified PDGF in equivalent doses (500 ng/ml) resulted in a large increase in new connective tissue formation, total DNA content, protein synthesis, and production of an extracellular connective tissue matrix. In addition, when wounds were treated with pure IGF-1 and purified PDGF connective tissue proliferation increased, approximating that seen in wounds treated with partially purified PDGF. This effect was significantly greater than that observed when using pure PDGF or IGF-1 alone indicating a synergistic effect of these growth factors in vivo. In conclusion, although most investigation of PDGF has been performed in vitro using tissue culture systems, it is interesting to extrapolate these findings to the in vivo environment to point out the possible physiologic role for PDGF. It is currently felt by most authors that PDGF is the primary growth factor involved in wound healing and repair reacting synergistically with other factors present in the blood (ie. IGF-1).

2. Epidermal Growth Factor (EGF). Epidermal growth factor represents one of the first growth factors described and to date, is foremost among those characterized. The existence of EGF was elucidated

when it was observed that extracts of submaxillary gland of the mouse injected into newborn animals induced precocious eyelid opening and incisor eruption due to direct stimulation of epidermal growth and keratinization (Cohen, 1962; Cohen and Elliott, 1963; Cohen, 1965). Epidermal growth factor was subsequently isolated and determined to be the factor responsible for these effects (Cohen, 1962). Originally EGF was isolated from mouse submaxillary gland (Cohen, 1962). It was found to be a single polypeptide chain, accounting for 0.5% of the protein content of the male mouse submaxillary gland. Recently, human EGF has been identified, isolated, and found to have all of the biological activities previously described for mouse-derived EGF (Cohen and Carpenter, 1975; Starkey et al., 1975). In addition, Gregory (1975) discovered that B-urogastrone, a gastric antisecretory hormone isolated from human urine, has nearly an identical amino acid sequence to human EGF and identical biologic activity suggesting that EGF has multiple effects in vivo.

Since its identification, EGF has been isolated and quantitated in a variety of human fluids. The levels of human EGF have been shown to be 5-17 ng/ml in saliva, 29-272 ng/ml in urine, 80 ng/ml in breast milk, and 2-4 ng/ml in blood (Carpenter, 1978). A more recent investigation by Oka and Orth (1983) determined that the concentration of EGF in platelet-rich plasma/serum was approximately 0.3 ng/ml compared to a platelet-poor plasma concentration of <0.015 ng/ml. This indicates that human platelets are a source of EGF activity. Similar to other growth factors (ie. PDGF, TGF-beta), sequestration and transport in platelets may provide a mechanism by which EGF is deposited at an injured site. Since EGF is released in plasma during coagulation it may be an important factor in the repair of

tissue damage (Oka and Orth, 1983). Currently the primary sites of EGF production in the human is not known.

For EGF to have an effect on a particular cell type it must first bind to receptors on the cell surface membrane. Specific, saturable receptors for EGF have been found on a number of ectoderm-, mesoderm-, and endoderm-derived cell types including human fibroblasts, bone-derived cells, vascular endothelial cells, corneal cells, glial cells, hepatocytes, and lens cells (O'Keefe et al., 1974; Hollenberg, 1975; Carpenter and Cohen, 1976; Gospodarowicz et al, 1977; Westermark, 1977; Gospodarowicz et al., 1978; Shupnik et al., 1980; Adamson and Rees, 1981; Ng et al., 1983b). Specifically fibroblasts have been found to contain 40,000 to 100,000 binding sites per cell with a dissociation constant of 2-4 x 10^{-10} (similar to PDGF) (Carpenter et al., 1975; Hollenberg and Cuatrecasas, 1975), indicating very tight binding between the EGF molecule and the cell membrane.

To date, EGF has been found to elicit a variety of biological responses in animals, organ cultures, and cell culture systems. Using a fibroblast tissue culture system, a variety of EGF mediated events have been identified which are ultimately responsible for increased cellular proliferation (Carpenter and Cohen, 1979). One of the early events is the active transport of low molecular weight compounds into the cell. Of particular importance to the wound healing environment are increased RNA, DNA and protein synthesis, along with increased cell proliferation which occurs following exposure to EGF. Addition of EGF to human fibroblasts for 12 hours has been shown to enhance RNA synthesis (Hollenberg and Cuatrecasas, 1973), while the addition of EGF to chick epidermis cultures

has resulted in a rapid increase in protein synthesis (Hoober and Cohen, 1967, Covelli et al., 1972). Increased DNA synthesis 20 hours after the addition of EGF to cell cultures has been reported for a variety of cell types including human fibroblasts, human endothelial cells, epithelial cells, glial cells, and hepatocytes (Armelin, 1973; Hollenberg and Cuatrecasas, 1973; Richman et al., 1976; Stoker et al., 1976; Westermark, 1976; Gospodarowicz and Mescher, 1977; Gospodarowicz et al., 1978). In most studies, DNA synthesis requires 1-2% serum in the culture medium and the presence of EGF for at least 5 hours (Carpenter and Cohen, 1976; Aharonov et al., 1978) while removal of EGF after DNA synthsis has begun (S phase) does not affect the rate of DNA synthesis (Carpenter and Cohen, 1979). Increased DNA synthesis has also been shown to be stimulated by EGF in several studies utilizing a bone organ culture system (Canalis and Raisz, 1979; Ng et al., 1983). Recently, EGF has been shown to act synergistically with insulin to increase DNA synthesis (Shipley et al., 1984) in a continuous clonal fibroblast cell line (AKR-2B). In the continuous presence of EGF (4 ng/ml) cell proliferation is also increased in human fibroblast cultures to the extent that many of the growth controlling mechanisms are altered. In the presence of EGF, fibroblasts continue to proliferate after cultures have become confluent, forming multilayered colonies unrestricted by density dependent inhibition of growth as is seen in normal cell cultures (Carpenter and Cohen, 1976). In contrast, Wrana and colleagues have shown that although EGF stimulates a small increase in fibroblast proliferation, it does not increase protein synthesis and may actually inhibit collagen synthesis (Wrana et al., 1986). Decreased protein synthesis has also been reported by other authors for

epithelial cells (Taketani and Oka, 1983), while collagen synthesis has been reported for bone derived cells (Canalis and Raisz, 1979).

Though the in vitro mitogenic activities of EGF have been well investigated, its actual in vivo role is less well defined. Using a rat model, Buckley and co-workers (1985) implanted polyvinyl alcohol sponges impregnated with EGF subcutaneously in the animal, with the EGF slowly released at a rate of 10-20 ug/day. The authors found that slow release of EGF resulted in a dramatic increase in the extent and organization of the granulation tissue at day 7 compared to the control animals. In addition, there was a doubling in the DNA content, a 33% increase in protein content, and an increase in collagen content. The authors concluded that EGF is an important component in soft tissue wound healing. Similarly, Schultz et al. (1987) found that EGF accelerated the rate of epidermal regeneration of partial thickness burns or split-thickness incisions in vivo; while Fabricant and colleagues (1982) found an increased rate of endothelium repair. Similarly, Lynch and co-workers (1987) found that EGF causes increased thickening of the epidermis, but does not affect connective tissue healing. Therefore, although the extent of EGF activity is not totally known, studies to date indicate that EGF affects a variety of cellular functions, and appears to play a vital role in the wound healing response.

3. Insulin-like Growth Factors (IGF) - Somatomedins. Another group of growth factors which have been investigated for their effect on cell proliferation and growth are the somatomedins or insulin-like growth factors, which were first described as a "sulfation factor" by Salmon and Daughaday in 1957 (Salmon and Daughaday, 1957). A variety of polypeptides

were previously investigated under the heading of insulin-like growth factors (IGF-I, IGF-II), somatomedins (SM-A, SM-C), and multiplication-stimulating activity (MSA). These growth factors have now been shown to actually consist of two major forms of insulin-like growth factor. Somatomedin-C and IGF I are identical peptides (Rinderknecht and Humbel, 1978a; Klapper et al., 1983) and can be designated as IGF I, while SM-A, MSA and IGF II have been found to be almost identical varying only slightly in chemical structure (Rinderknecht and Humbel, 1978b; Marquardt et al., 1981) and will be referred to as IGF II

IGF I has been purified from human serum and found to consist of a single chain of 70 amino acids with three internal disulfide bonds with a molecular weight of 7646 (Rinderknecht and Humbel, 1978a; Svoboda et al., 1980) . It is produced in response to circulating growth hormone and as a result may secondarily regulate growth hormone dependent skeletal formation. IGF II has been isolated from rat liver cultures and consists of 67 amino acids with three disulfide bonds and a molecular weight of 7471. More than half of the amino acid positions are identical in IGF I and IGF II and both exhibit considerable homology to insulin (Rinderknecht ahd Humbel, 1978b)

Although insulin-like growth factors are primarily produced in the liver, their biologic activity and tissue distribution is limited by a binding protein, thus making the local production of insulin-like growth factors by extra-hepatic cells physiologically important (D'Ecrole et al., 1984). Recent evidence indicates that other tissues are capable of producing these growth hormones. Human fibroblasts (Atkinson et al., 1980, Clemmons et al., 1981), cartilaginous tissue (Bennington et al., 1983) and

bone organ cultures (Stracke et al., 1984) have all been found to produce increased amounts of insulin-like growth factors. IGF I which is synthesized by the liver probably acts as a circulating hormone, while that which is synthesized in other tissues may act as a local growth factor (Canalis, 1985). The synthesis of IGF I by the liver and peripheral tissues is felt to be regulated by pituitary growth hormone (GH) and as such the effects of GH are felt to be mediated by IGF I (Trippel et al., 1983; Schoenle et al., 1982). However, IGF I is not solely growth hormone-dependent, with cortisol, insulin, and thyroid hormone all providing regulatory roles (Schalch et al., 1979; Schwander et al., 1983; D'Ecole et al., 1984). Similarly, IGF II is also synthesized primarily in the liver (Dulak and Temin, 1973; Moses et al., 1980b) with peripheral production having been identified in fibroblasts (Adams et al., 1983a; Adams et al., 1983b). Although the concentration of IGF II is three to four times higher in normal individuals than the concentration of IGF I, IGF II is far less responsive to growth hormone (Van Wyk, 1984). In addition, the concentration of IGF II is higher in fetal serum than in adult serum, suggesting a possible role for IGF II in embryonic growth (Moses et al., 1980a). Therefore, current information indicates that there are probably two forms of insulin-like growth factors; however, the exact role of each has not yet been defined.

One of the biological effects of insulin-like growth factors which has been widely investigated is its effect on cell proliferation. Specific receptors for insulin-like growth factors have been found in nearly all tissues from both fetal and adult animals (D'Ercole et al., 1976; Sara et al., 1983; Van Wyk, 1984). In addition, IGF I has been found to stimulate

DNA synthesis, with or without subsequent cell proliferation, in a wide variety of species and cell types, including fibroblasts, epithelial cells, smooth muscle cells, hepatic cells and hematopoietic cells (Van Wyk, 1984; Clemmons, 1985). When present in hyperphysiologic concentrations insulin can produce somatomedin-like effects by binding to the IGF receptor (Hayashi et al., 1978); however, at physiologic concentrations insulin does not appear to have the growth promoting activity of the insulin-like growth factors (Bolander et al., 1981). Essentially, IGF I effects cell proliferation by acting within the cell cycle as a progression factor. By itself in physiologic concentrations, IGF I does not duplicate the growth promoting activity of serum. As previously discussed, Pledger and colleagues (1977) demonstrated that the sequential action of PDGF and IGF was synergistic in stimulating DNA synthesis in quiescent fibroblasts. Van Wyk and co-workers (Russell et al., 1984; Van Wyk et al., 1984) have demonstrated that the progression activity of platelet-poor plasma from normal donors can be almost totally repressed if a monoclonal antibody specific for IGF I is incorporated into the culture medium. The effects of insulin-like growth factors have been further defined by demonstrating that EGF and IGF I can completely substitute for plasma in cultures of BALB/c 3T3 fibroblasts during the progression phase of the cell cycle.

In addition to the effect on cellular proliferation of fibroblasts, insulin-like growth factors have also been shown to have a variety of effects on other cell types (Van Wyk, 1984). In cartilage, insulin-like growth factors have been shown to stimulate proteoglycan, DNA, RNA, collagen and non-collagenous protein synthesis. It is also felt to play a role in collagen synthesis and proliferation in bone cells (Canalis, 1985).

With regard to the effects on muscle, insulin-like growth factors have been shown to increase glycolysis and glycogen synthesis (Poggi et al., 1979), as well as increase DNA and protein synthesis (Salmon and DuVall, 1970). In adipose tissue, IGF I mimics all the effects of insulin including glucose oxidation, lipid and glycogen synthesis, and inhibition of epinephrine-stimulated lipolysis (Van Wyk, 1984).

From a systemic standpoint, the in vivo effects of insulin-like growth factors can essentially be broken into insulin-like effects and growth-promoting effects. For example, insulin-like growth factors have been shown to produce a prolonged lowering of the blood sugar and stimulation of glucose incorporation into glycogen (Olez et al., 1970). The ability of insulin-like growth factors to stimulate growth has been shown by a number of investigators using a variety of animal models (Van Buul-offers and Van den Brande, 1979; Rothstein et al., 1980; Schoenle et al., 1982a; Schoenle et al. 1982b). In these experiments, IGF I and IGF II have been added exogenously to normal and hypophysectomized animals with a resulting increase in total body weight. The physiologic significance of these findings, above the effects of endogenously produced insulin, have not been completely determined.

Overall, from the standpoint of wound healing, insulin-like growth factors, particularly IGF I are felt to play a necessary local role in DNA synthesis and subsequent cell proliferation as a progression factor in blood. It is through the interraction with PDGF and EGF that IGF I is felt to have its major effect. In addition, there are a variety of other systemic and local effects in which insulin-like growth factors play an essential role in cellular physiology.

4. Transforming Growth Factor-Beta (TGF-beta). Transforming growth factors have been defined as hormone-like polypeptides that reversibly induce certain nonneoplastic indicator cells to express the malignant phenotype which is measured in vitro by the ability of anchorage-dependent indicator cells to grow in soft agar under anchorage-independent conditions (De Larco and Todaro, 1978; Todaro et al., 1981; Roberts et al., 1983b). Two classes of transforming growth factos have been identified on the basis of their relationship to EGF (Roberts et al., 1983a) Transforming growth factor-alpha (TGF-alpha) competes with EGF for binding sites, has a similar amino acid sequence to EGF and is functionally identical to EGF. Transforming growth factor-beta (TGF-beta) is an entirely different polypeptide with a different functional activity. TGF-beta has been purified by several investigators and found to be a dimer (M.W.- 25,000 daltons) which is resistant to degradation at elevated temperatures (95^{o}C) and acidic conditions (Sporn et al. 1986). The TGF-beta dimer is composed of nearly identical 12,500 dalton subunits of 112 amimo acids held together by disulfide bonds (Assoian et al., 1983; Frolik et al., 1983; Roberts et al., 1983; Massague, 1984), with only the non-reduced molecule showing biological activity (Sporn et al., 1986). The amino acid sequence of human (Roberts et al., 1983a) and mouse (Derynck et al., 1986) TGF-beta has been identified. Currently, TGF-beta has been isolated from a number of neoplastic and nonneoplastic tissues including bovine kidney, human placenta, human platelets, fetal rat calvaria, and bone (Childs et al., 1982; Assoian et al., 1983; Frolik et al., 1983; Roberts et al., 1983b; Centrella and Canalis, 1985; Hauschka et al., 1986).

Similar to other polypeptide factors, TGF-beta tightly binds to a specific cell membrane receptor (Kd = $6x10^{-11}$ to $1x10^{-12}$). Unlike other polypeptide factors, the receptor for TGF-beta is found on virtually all cells, normal or malignant, including epithelial cells, mesenchymal cells, and hematopoitetic cells such as lymphocytes (Frolik et al., 1984; Tucker et al., 1984; Massague and Like, 1985; Sporn et al., 1986; Wakefield et al., 1987). There is no known cross reactivity between TGF-beta and the membrane receptors for other growth factors, and other growth factors do not bind to the membrane receptor for TGF-beta. Unlike other polypeptide factors, the receptor for TGF-beta does not show extensive down regulation following exposure to high concentrations of TGF-beta (Frolik et al., 1984; Massague, 1985).

Platelets and bone are the major storage sites for TGF-beta in the body. Assoian and colleagues (1983) have found that platelets contain 40-100-fold more TGF-beta than other non-neoplastic tissues. Seyedin and co-workers have isolated TGF-beta (formerly called cartilage-inducing factor-A) from bovine demineralized bone (Seyedin et al., 1985; Seyedin et al., 1986) and Robey and colleagues (1987) have isolated TGF-beta from fetal bovine bone cells. The fact that TGF-beta is released from platelets upon clotting (Assoian and Sporn, 1986), in conjunction with the known role of platelets in wound healing is felt to support the hypothesis that the major physiological role of TGF-beta is to facilitate tissue repair and regeneration. In addition, TGF-beta has recently been shown to be secreted from activated macrophages and peripheral blood monocytes (Assoian et al., 1987), lending further support to its role in repair and regeneration.

Transforming growth factor-beta is currently felt to be a regulatory molecule acting by autocrine and paracrine mechanisms, with an important role in the function of epithelial cells, connective tissue cells and cells of the immune system (Sporn et al., 1986). An important aspect of the proposed biologic roles of TGF-beta is that it is bifunctional. This means that it can either stimulate or inhibit proliferation, differentiation, or other cell processes depending on the target cell and the presence of other specific growth factors. Initially, TGF-beta was felt to be an inhibitor of cell proliferation since it exerted this effect in multiple tissue culture systems including: T and B lymphocytes, fibroblasts, hepatocytes, and keratinocytes (Sporn et al., 1986). In cells of mesenchymal origin, TGF-beta stimulates or inhibits proliferation depending on the other growth factors present (Assoian et al., 1985; Moses et al., 1985). Recently Roberts and co-workers demonstrated the multifunctional role of TGF-beta using Fischer rat 3T3 fibroblasts transfected with a cellular myc gene. Using a soft agar assay system they found that in the presence of PDGF, TGF-beta had a proliferative effect, while in the presence of EGF, identical concentrations of TGF-beta had an inhibitory effect (Roberts et al., 1986). These results are similar to those obtained by Rizzino and co-workers (1986), who found that in the presence of EGF, TGF-beta caused an increased response to PDGF by NRK cells, while in the absence of EGF, TGF-beta caused a decreased cellular response to PDGF. Slightly different results were obtained by Fernandez-Pol and co-workers (1986) using a tissue culture system and a variety of clonal cell lines. These investigators found that the cellular response to EGF and TGF-beta was dependent on the culture conditions. In the presence of 5% serum, TGF-beta and EGF

inhibited cell proliferation. In contrast, under serum-free conditions (ie. in the absence of PDGF), TGF-beta, in the presence of EGF, induced DNA synthesis and cell proliferation in growth-arrested cellular monolayers. However, TGF-beta and EGF were unable to sustain cell proliferation beyond the third or fourth round of cell division. When used alone, TGF-beta had only a mild stimulatory effect on cell proliferation. The authors concluded that TGF-beta requires the presence of other growth factors such as EGF, IGF, and PDGF to induce full and persistent progression of cells through the cell cycle. The necessity of multiple growth factors for maximum proliferation had previously been shown by Assoian et al. (1984), who found that to achieve maximum DNA synthesis in NRK fibroblasts, PDGF, EGF and TGF-beta needed to be present simultaneously in the growth medium.

In contrast to the bifunctional effects of TGF-beta which have been previously reported, Wrana and his colleagues (1986) recently found that TGF-beta has only a stimulatory effect in fibroblasts with regard to proliferation, DNA synthesis, and protein synthesis. Unlike previous investigators who have utilized established cell lines exhibiting immortal, non-neoplastic phenotypes and growing indefinitely in culture, Wrana et al. utilized normal human diploid gingival fibroblasts, having a finite lifespan to more closely duplicate an in vivo situation. Although TGF-beta alone (1.0 ng/ml) did not simulate cell proliferation, the addition of EGF (2.5 ng/ml) to subconfluent quiescent fibroblast cultures in serum-free medium resulted in a significant (75%) increase in cell number. This response was significantly reduced in confluent cell cultures. In addition, protein synthesis was significantly increased with initial stimulation evident within three hours of administration of TGF-beta and

reaching a maximum after 24 hours. The authors concluded that the stimulatory effect of TGF-beta on human gingival fibroblasts is consistent with a physiological role in the wound healing response (Wrana et al., 1986).

In addition to its effect on cell proliferation and protein synthesis, TGF-beta exerts a variety of other effects on cell function. These include the stimulation of glucose production, amino acid transport, and glycolysis in fibroblasts (Boerner et al., 1985; Inman and Colowick, 1985; Racker et al., 1985). TGF-beta has also been shown to accelerate protein, collagen, and DNA synthesis when injected along with EGF into experimental wound healing chambers in the backs of rats (Sporn et al., 1983). Using a mouse model system, Roberts and colleagues (1986) report that when injected subcutaneously, TGF-beta causes a rapid local increase in angiogenesis and collagen production. TGF-beta has also been shown to enhance prostaglandin release in calvaria organ cultures (Tashjian et al., 1985), and block natural killer function in lymphocytes (Kehrl et al., 1986). Recently Wahl and her co-workers (1987) demonstrated that TGF-beta is capable of inducing chemotaxis in human peripheral blood monocytes. This would further support the role of TGF-B in wound healing and regeneration. In general, the action of TGF-beta, both in vitro and in vivo, on a wide variety of cell types suggests to many investigators that TGF-beta has a very important role in the body's tissue repair and wound healing response.

E. Statement of Problem

The majority of current regenerative periodontal procedures are unpredictable in achieving a new periodontal attachment. This is due in

large part to the inability of cementoblasts, fibroblasts, and osteoblasts to form a new attachment apparatus before the junctional epithelium migrates apically. However, the potential for regenerating periodontal structures is clearly present. The recent use of the millipore filter is encouraging, but it too appears to have limitations. It can be seen that the majority of investigators have attempted to regenerate a new periodontal attachment through a mechanical manipulation of the involved tissues. Another approach to this problem is by investigating the biologic mechanisms which regulate the activity of the cell types present in a regenerative or wound healing environment and to then develop strategies to regulate and maximize the body's natural regenerative capacity. Current studies have shown that wound healing and regeneration are largely controlled by locally produced chemical mediators. Initially, chemotactic factors control the migration of cells which are present in the local ennvironment to aid in healing or repair. Growth factors then control the growth and proliferation of these cells which is followed by the formation of an extracellular matrix and ultimately repair or regeneration of the wound.

Regeneration of a periodontium which has been lost due to disease would also be expected to respond in the same manner as other "wounds" in the body. As such, much of the information which has been obtained regarding the mechanisms which control healing and regeneration can now be applied to regeneration of the periodontium. Currently, this approach to has not been widely utilized. Strategies need to be devised to study the growth factor response to cell types present in the periodontium. This information can then be used to "direct" the basic biologic events and

improve periodontal regeneration. Therefore, the purpose of this study was to isolate cell populations from adult human alveolar bone, gingival connective tissue, and the cemental aspect of the periodontal ligament, and to the characterize these cell populations using specific morphologic and biochemical criteria. These cell populations would then be used to investigate the effects of specific paracrine growth factors on cellular proliferation.

III. MATERIALS AND METHODS

A. Tissue Explants

1. Collection of Tissue Samples. The experimental protocol for obtaining tissue samples was submitted to the Institutional Review Board and determined to be exempt under DHHS Regulation 46.101(b)(5) for research involving the collection of diagnostic and pathologic specimens. All tissue samples were obtained from healthy adult volunteers who were undergoing dental treatment at The University of Texas Health Science Center at San Antonio and were handled under sterile conditions. All samples were taken from the surgical site and placed directly into sterile 50 ml tissue culture tubes containing a sterile solution of Dulbeco Modified Eagle's Medium (DMEM) and a 1% antibiotic solution of penicillin (100 U/ml) and streptomycin (100 ug/ml). All samples were immediately taken to the laboratory for explanting. All subsequent steps were accomplished in a laminar flow hood to maintain a sterile environment.

2. Periodontal Ligament Explants. Cells derived from periodontal ligament were obtained in a manner similar to that described by Blomlof and Otteskog (1981). All samples were obtained from impacted third molar teeth with no apparent pathology which were removed for elective reasons in the Department of Oral Surgery. Following extraction, the teeth were transported to the laboratory where they were rinsed five times in fresh DMEM supplemented with a 5% antibiotic solution to decrease the likelihood of sample contamination. Using a sterile #11 Bard Parker scalpel blade all adherent connective tissue was removed from the crown and the coronal one-third of the root and discarded, to preclude contamination from these

tissues. The periodontal ligament and adjacent cementum was then scraped from the middle one-third of each root and placed in a 25 cm^2 tissue culture flask. The tissue sample was allowed to dry in an incubator at 37°C for five minutes to facilitate attachment to the bottom of the flask. Culture medium consisting of DMEM supplemented with 10% Fetal Bovine Serum (FBS) and a 1% antibiotic solution was added and the tissue sample was incubated under standard conditions in a humidified environment at 37°C in a atmosphere of 5% CO_2 and 95% O_2. The flasks were left for three to four weeks with media changes accomplished every three days. On days 1, 7, and 14 the cells were incubated for twenty-four hours in culture medium containing five times concentrated antibiotics to decrease the likelihood of contamination. The periodontal ligament/cementum cells were allowed to migrate from the tissue and when the initial explant reached 60% confluence in a 25 cm^2 tissue culture flask, the cells were transferred from the flask. The existing culture medium was evacuated and the cells were washed with 3 ml of Hank's Balanced Salt Solution (HBSS) for approximately one minute. The cells were then removed from the flask using a mixture of 50% trypsin (0.025%)-EDTA (0.2%) solution (GIBCO) and 50% HBSS. The cells were observed under a Phase Contrast Microscope (Olympus) for detachment and immediately placed in a 75 cm^2 tissue culture flask containing 10 ml of culture medium (10% FBS/DMEM) which inactivated the action of the trypsin mixture. These cells were designated passage one. Tissue culture medium was changed at 72 hour intervals and the cells were allowed to grow to confluence. When confluence was reached the detachment procedure was repeated and the cells were split at a one to three ratio and replated in 75 cm^2 tissue culture flasks under standard conditions.

Twenty-one periodontal ligament/cementum explants were initiated. One became contaminated and had to be discarded. From the remaining 20 samples only two produced viable cell populations. These were designated PL4, which was established from an 18 year old white male donor, and PL7, which was established from a 21 year old white female donor. These periodontal ligament/cementum cell populations were then utilized in these experiments.

3. Bone Explants Bone-derived explants used in these studies were initiated from alveolar bone and long bones in a manner similar to that described by Beresford et.al. (1983a, 1983b) Human bone was obtained from adults with no malignancy or metabolic disease. All bone-derived explants were obtained from sub-periosteal human trabecular bone.

a. Alveolar Bone. Alveolar bone was harvested from third molar extraction sites and transported to the laboratory in a similar manner described for periodontal ligament/cementum samples. The bone samples were rinsed in an antibiotic solution as previously described and then scraped vigorously with a sterile #11 Bard Parker blade to remove any attached soft tissue or periosteum. The bone samples were then broken into 2-4 mm pieces and placed in a 25 cm^2 tissue culture flask. After five minutes, culture medium was added and the cells were incubated as previously described for the periodontal ligament/cementum samples. Cell populations were allowed to migrate from the tissue and the cells were handled in the same manner as that described for the periodontal ligament/cementum cell populations.

One explant of alveolar bone cells was established from an 18 year old male. This cell population was designated BP1 and was utilized in the majority of the experiments.

b. Long Bones. Cell populations derived from human adult long bones were were obtained from Dr. Alex Valentin-Opran in the Department of Medicine, Endocrine Division, The University of Texas Health Science Center. The explant technique was essentially the same as previously described. This cell population was designated LB1 and was utilized only in experiments where multiple growth factors were used.

4. Gingival Connective Tissue Explants. Two fibroblast populations were established from clinically healthy gingiva as described above, except that dense gingival connective tissue was diced into 1-2 mm^3 pieces before placement in 25 cm^2 flasks. Explant GF2 was obtained from a healthy 20 year old male and was utilized in all characterization experiments and a portion of the growth factor experiments. Explant GF6 was obtained from a healthy 31 year old male and was utilized in all growth factor experiments.

B. Handling of the Cell Populations

Following the establishment of cell populations from the periodontal ligament/cementum, alveolar bone, long bones, and gingival connective tissue the cells were stored at -70°C prior to use in the study. Flasks of cells were slowly frozen at -70°C in 10% DMSO / 30% FBS / and 60% DMEM using Cole Parmer Cryotubes. Cells from passage one through five were frozen so as to store enough cells for subsequent experiments. Cells were not used if they had been passaged more than five times.

Prior to experimentation, the cell populations to be used were rapidly thawed in a water bath to 37°C, placed in 75 cm^2 tissue culture flasks with 10% FBS/DMEM culture medium without antibiotics and incubated under standard conditions. All cell cultures were allowed to grow to confluence

and passaged at least once prior to characterization and growth factor assays. Tissue culture medium was changed every 3-4 days to maintain viable cell cultures. If a culture became contaminated it was immediately removed from the incubator and the remaining cell populations carefully observed for contamination.

At the start of all experiments the cells were plated at a specific density which varied for each protocol. Plating density was achieved in the following manner and was the same for all experiments. Cell populations in 75 cm^2 flasks, which had just reached confluence, or were slightly subconfluent, and in which the tissue culture medium had been changed within 24 hours, were selected for assay. The cells were rinsed twice with PBS and then trypsinized as previously described. The cell suspension was then placed in 2 ml of DMEM supplemented with 10% FBS to inactivate the trypsin. The cell suspension was thoroughly mixed using repeated aspiration with a pipette and 0.1 ml of the cell mixture was removed and counted using a hemocytometer. The number of cells/ml was determined by counting 10 randomly chosen fields from the hemocytometer and multiplying the number by 1,000. The appropriate volume of cell suspension was then removed to provide the total number of cells necessary for the assay and added to the appropriate volume of tissue culture medium. Finally, 1 ml of the cell suspension was added to each well of a 24-well tissue culture plate which was to utilized in that particular assay. A volume of 0.2 ml was added when 96-well tissue culture plates were used.

C. Cell Characterization

All experiments were duplicated to verify the accuracy of the results.

1. Morphologic Analysis. Prior to biochemical cell characterization, the cellular morphology was examined under phase contrast microscopy.

2. Cyclic-AMP Assay. The response to bovine parathyroid hormone (bPTH) was assessed by measuring intracellular c-AMP levels as described by Luben, Wong and Cohn (1976). All assays were accomplished in triplicate to allow for statistical analysis. Cells were plated at confluence in 24 well plates in tissue culture medium and allowed to recover for 24 hours. Prior to stimulation, the tissue culture medium was removed and following a 1 minute rinse with sterile HBSS, the cultures were incubated for six hours in 0.2% bovine serum albumin in DMEM. This was accomplished to quiesce the cells so that all cell populations were at the same reduced level of activity prior to stimulation. The medium was then removed and the cells were rinsed once with DMEM. Next, medium containing 500 ng/ml (125 nM) of bovine 1-34 PTH synthetic peptide, 1mM isobutylmethylxanthine (IBMX), and 10^{-7}M forskolin in HBSS was added to half the wells. Control cells received the same solution without bPTH. Isobutylmethylxanthine was used to prevent the chemical breakdown of cAMP. Forskolin was used in order to optimize conditions for PTH-dependent stimulation of c-AMP. Low doses of forskolin has been shown to potentiate hormone induced adenylate cyclase activity through enhanced guanine nucleotide binding with minimal increases in basal levels of c-AMP (Seamon and Daly, 1981). The use of forskolin has previously been shown to have a synergistic effect on PTH modulation of c-AMP in osteoblast cultures (Henry, Cunningham & Noland 1983, Henry 1985). In preliminary experiments varying concentration of bPTH ranging from 100 ng/ml to 750 ng/ml was utilized. The cells were incubated for 15 minutes

at 37°C and then immediately placed on ice to stop the reaction. The medium was removed and the cells were rinsed three times with ice cold Phosphate Buffered Saline (PBS).

Intracellular c-AMP was obtained using an acid/ethanol extraction procedure. A 0.5 ml volume of an ice cold 95% Ethanol/20mM HCl solution was added to each well and the plates were placed at -20°C for 18 hours. At the end of this time period the acid/ethanol solution was removed and placed in a 12 x 75 mm borosilicate tube. The wells were each rinsed once with 0.5 ml of the acid/ethanol solution which was added to the cell extract. Cell extracts were dried in a boiling water bath and then reconstituted using 500 ml of sodium acetate buffer supplied in the Immunonuclear c-AMP radioimmunoassay kit. All samples were diluted 1:2 with buffer prior to assay amd cyclic-AMP levels were determined using the plasma assay protocol of the radioimmunoassay kit obtained from the Immuno Nuclear Corporation.

3. Alkaline Phosphatase Assays. Cellular alkaline phosphatase was examined using three different protocols. The response to bovine parathyroid hormone (bPTH) was measured following a 15 minute and a 48 hour exposure while the alkaline phosphatase response to 1,25 dihydroxyvitamin D was measured following a 48 hour exposure to the hormone.

4. Parathyroid Hormone Assay. - Fifteen Minute Stimulation. Cellular alkaline phosphatase response to bPTH was initially measured similar to the procedure reported by Luben, Wong and Cohn (1976), except that a 15 minute rather that a 48 hour exposure to the hormone was used. All samples were assayed in quadruplicate to allow for statistical

analysis. Cells were plated at confluence in 24-well tissue culture plates and allowed to recover overnight in tissue culture medium without antibiotics. The cells were rinsed once with sterile PBS prior to stimulation to remove residual culture medium. The experimental wells were then exposed to media containing 500 ng/ml of bPTH and 1mM isobutylmethylxanthine in HBSS for 15 minutes at 37°C. The control cells were incubated under identical conditions minus the bPTH. Following incubation the plates were immediately placed on ice. The media was removed and saved for assay, while the cells were rinsed three times with ice cold PBS. Cells were lysed to obtain the intracellular alkaline phosphatase by freeze-thawing three times in 0.1 ml distilled water followed by the addition of 0.1 ml of 2% Triton X-100. The solubilized cell lysates were combined with previously obtained stimulation medium and assayed for alkaline phosphatase content.

Alkaline phosphatase was measured by the addition of 100 ul of sample to a solution contaning 20 ul of 2mM diethanolamine-HCl (pH 10), 20 ul of 10 mM $MgCl_2$, 29 ul of 20mM paranitrophenolphosphate, and 40 ul of distilled water. Blanks contained all of the above except the sample. The reaction mixture was incubated in a 37°C water bath and allowed to proceed until a yellow color became evident. The reaction was then stopped simultaneously in all samples by the addition of 1 ml of 1 M NaOH. The relative amount of alkaline phosphatase was measured using a spectrophotometer at 410 nm.

5. Parathyroid Hormone Assay - Forty-Eight Hour Incubation. Cells were plated at either 2,000 cells/well or 9,000 cells/well in tissue culture medium without antibiotics using 96-well tissue culture plates. All samples were run in quadriplicate to allow statistical analysis of the

results. The cells were then allowed to incubate for 72 hours to establish confluent and sub-confluent cell populations. This was followed by a single rinse with sterile phosphate buffered saline (PBS) and incubation in DMEM supplemented with 0.1% bovine serum albumin (BSA) with and without bPTH, with multiple concentrations of bPTH (10 ng/ml, 100 ng/ml) utilized. Following a 48 hour incubation at 37°C under standard conditions the cells were rinsed three times with ice cold PBS. The cell membranes were solubilized by the addition of 50 ul/well of 1% Triton X-100, using gentle aggitation for 20 minutes. A volume of 50 ul/well of assay buffer was then added to each well to determine the relative amount of alkaline phosphatase. Assay buffer was composed of 0.4mM diethanolamine-HCl (pH 10), 2mM $MgCl_2$, and 4mM paranitrophenolphosphate. The reaction was allowed to proceed for approximately 30 minutes until a yellow color began to appear. The reaction was then terminated in all wells by the addition of 150 ul of 1 N NaOH. The relative amount of alkaline phosphatase was then determined using a spectrophotometer at 410 nM.

6. 1,25 Dihydroxyvitamin D3 Assay. The cellular alkaline phosphatase response to 1,25 $(OH)_2$ Vitamin D was measured in an identical manner to that described for the 48 hour response to bPTH with the following exceptions. First, the cells were plated at 2,000 cells/well and 6,000 cells/well to establish confluent and sub-confluent cell populations. Next, the following concentrations of 1,25 $(OH)_2$ Vitamin D were used: 2.4×10^{-8}M and 2.4×10^{-9}M. All other aspects of the protocol were identical to the previous description.

D. Growth Factors

Purified grade growth factors were used in all studies. PDGF was generously donated by Dr. Harry Antoniades. TGF-Beta was generously donated by Dr. Michael Sporn. Recombinant IGF-1 was purchased from Angen Biologicals. EGF was purchased from Collaborative Research. Platelet-poor plasma and fetal bovine serum were purchased from K.C. Biologicals.

E. Cell Proliferation

Cell populations were assayed for proliferation as described by Graves et al. (1983), with the results confirmed in multiple experiments. Within an individual experiment each test and control sample was tested in duplicate. In addition, all of the cell populations in a given assay were handled simultaneously under identical conditions. The cell populations to be used varied depending on the experiment being performed. Initially only platelet-derived growth factor (PDGF) was used in the experiments and the response was examined with all of the cell populations. Subsequently, epidermal growth factor (EGF), transforming growth factor-beta (TGF-Beta), and platelet-derived growth factor were assayed individually and in combinations. The experimental protocol used was essentially the same for all of the assays.

Cell populations were plated at subconfluence (15,000 to 25,000 cells) in 24-well tissue culture plates and incubated in 0.5 ml of DMEM supplemented with 10% FBS for 12 hours at 37°C, 5% CO_2. This initial period allowed for the cells to attach to the tissue culture plates and recover from the plating procedure. At day 0 the cells were rinsed twice in warm PBS and changed to DMEM containing the indicated concentration of platelet-poor plasma. Growth factor(s) was(were) then added singly or in

combination to each well at appropriate concentrations (see figure legends). The proliferative response to growth factors was compared to a negative control consisting of cells incubated in DMEM supplemented with platelet-poor plasma alone, and a positive control consisting of cells incubated in DMEM supplemented with 10% FBS. The positive control of DMEM supplemented with 10% FBS has been shown to provide an enriched supplement of growth factors and nutrients which has been found to be highly stimulatory for cells of mesenchymal origin. The media were changed every 3 or 4 days and at that time the indicated cells were trypsinized and counted using a hemocytometer as previously described. Cell number was expressed as the number of cells/ml.

F. DNA Synthesis

DNA synthesis was measured using two different protocols. Both autoradiography and acid insoluble ^{3}H Thymidine incorporation were used to determine the change in DNA synthesis following exposure of the cell populations to growth factors.

1. Autoradiography. DNA synthesis was measured by autoradiography in the following manner. Cells were plated at approximately 10,000 cells/well in 96-well microtiter plates and grown to confluence DMEM supplemented with 10% FBS. The cells were then depleted and assayed simultaneously following two slightly different protocols. Depletion is necessary to allow cells to become quiescent so that the measured response will be due solely to the test medium rather than the initial tissue culture medium in which the cells grew to confluence.

One group of cells was depleted for 72 hours in DMEM supplemented with 3% platelet-poor plasma. At the end of the depletion period, the cells were rinsed and tested in fresh identical medium. The other group of cells were depleted for 72 hours in DMEM supplemented with 0.1% Bovine Serum Albumin, insulin (5Ug/ml) and transferrin (5Ug/ml), rinsed and tested in fresh identical medium. The concentrations of insulin and transferrin used have been shown to enhance the response of Balb c/3T3 cells to platelet-derived growth factor while minimally stimulating DNA synthesis (Rockwell, 1984; Van Wyk, 1984). In addition, this concentration of insulin is sufficiently high to enable insulin to bind to the insulin-like growth factor 1 receptor (Van Wyk, 1984). Varying concentrations of PDGF were then added to the media and the cells allowed to incubate for 18 hours. The positive control for these experiments was DMEM supplemented with 10% FBS and the negative control was the depletion medium without growth factors. After 18 hours the cells were pulse labeled for 24 hours by the addition of ^{3}H thymidine (5 UCi/ml, specific activity 48 UCi/mMole) to the culture medium.

At the end of the pulse labeling period the cells were immediately fixed using a methanol fixation procedure. The microtiter plates were placed on ice and carefully rinsed once with ice cold saline and then twice with ice cold methanol. Ice cold methanol was then added to the microtiter plates and allowed to remain on the cells for 15 minutes. This was followed with one rinse using ice cold methanol and four rinses using ice cold deionized water. The plates were then dried prior to autoradiographic processing.

The labeled nuclei were then identified by placing a thin layer of photographic emulsion over the cells. The emulsion reacts with the labeled nuclei and producing a blackening of nuclei. This allows identification of nuclei which were metabolically active during the course of the procedure. Kodak NTB2 emulsion was selected for use in these experiments since it is recommended for low and medium energy beta emitters and gamma emitters, and is sensitive enough to record electron tracks with energies less than 0.2 MeV. This level of sensitivity has been shown to produce good grain density with low levels of background fog. All of the autoradiographic procedures were performed in absolute darkness to avoid exposure of the emulsion material. Kodak NTB2 emulsion was mixed with 42°C deionized water in a ratio of 1 gram of emulsion to 1 ml of water. The mixture was vortexed and placed in a 42°C water bath to maintain constant temperature and allow the emulsion to remain in solution. Next, 100 ul of emulsion was added to each well, allowed to remain for approximately 1 minute and then removed. A thin film of emulsion without bubbles, which totally covered the cells, was left on the bottom of the well. The microtiter plate was then placed in 3 layers of aluminum foil to exclude any light and placed at 4°C for seven days. At the end of the seven day period the plate was again taken to the darkroom and developed in the following manner. First, 100 ul of Kodak Dektol developer (1 to 2 dilution) was added to each well and allowed to remain for four minutes. The plate was then rinsed for ten seconds with distilled water and 100 ul of Kodak fixer was added to each well and allowed to remain for 5 minutes. The plate was rinsed for five minutes with distilled was and placed in a dust-free atmosphere to dry. Nuclear staining was verified under phase contrast microscopy.

Cell membranes were stained to assist visualization of the total number of cells. This allows differentiation of cells which have stained and unstained nuclei, thus providing a percentage of labeled nuclei. Hemotoxylin stain was added to each well and allowed to stand for fifteen minutes. This was followed by a 1 minute rinse with deionized water and verification of staining under phase contrast microscopy. The total number of labelled and unlabelled cells were then determined for three high powered fields and the average of each provided the percentage of labelled cells per well. All experimental samples were run in triplicate.

2. Acid Insoluble 3H Thymidine Incorporation. DNA synthesis was measured in a manner similar to that described by Graves et al. (1983). Cells to be used in the experiment were again plated at approximately 10,000 cells/well in DMEM supplemented with 10% FBS and allowed to grow to confluence. The cells were then quiesced using the same depletion protocols as previously described with the following exceptions. In experiments using only PDGF a 48 hour rather than a 72 hour depletion period was used for cells incubated in insulin and transferrin. In experiments using multiple growth factors (PDGF, EGF, IGF, and TGF-beta) the cells were depleted in 1% platelet poor plasma for 48 hours. The depletion medium was removed and growth factors added singly or in combinations depending on the experimental protocol (see figure legends) to the negative control medium utilized in that protocol. In experiments using only PDGF the growth factor was added to either DMEM supplemented with 0.1% Bovine Serum Albumin, insulin (5Ug/ml) and transferrin (5Ug/ml), or DMEM supplemented with 3-4% platelet poor plasma depending on the depletion medium used. In experiments using PDGF, EGF, IGF and TGF-beta,

the growth factors were added to DMEM supplemented with 0.1% albumin. The positive control for all DNA synthesis experiments was DMEM supplemented witn 10% FBS. The cells were then incubated for 24 hours in the respective experimental and control media with 2.0-5.0 UCi/ml of ^{3}H Thymidine added to the assay medium for the last two hours. The cells were then rinsed twice with sterile PBS, placed on ice and fixed by the addition of ice cold 5% tricholracetic acid (TCA) for 20 minutes. The plates were then rinsed with ice cold 5% TCA followed by five rinses with ice cold dionized water. The fixed material was then solubilized using 1% sodium dodecyl sulfate (SDS) detergent. One hundred microliters of 1% SDS was added to each well and allowed to sit for 30 minutes. The radioactive mixture was removed and placed in a scintillation vial containing 5 ml of Hydrofluor liquid scintillation fluid (National Diagnostics, Sommerville, N.J.). Each well was rinsed with 200 ul of deionized water and the mixture was added to the scintillation vial and vortexed for 15 seconds to completely mix the material. Each vial contained the radiographic material from one well. The scintillation vials along with blanks containing only scintillation fluid were then counted on a Beckman LS-250 liquid scintillation counter. Values for each sample were reported as counts per minute with each experimental sample run in triplicate.

G. Analysis of Data

Results from light microscopy were descriptive and did not require statistical analysis.

Sample mean and standard error of the mean were determined for all data.

Statistical analyses were performed on the cell characterization data using a Statview 512 Software Package running on an Apple Macintosh Plus microcomputer. Within each experimental protocol the results for each cell type were analyzed using an Analysis of Variance (ANOVA). Scheffe's F-test was used as the post hoc test.

Data from the cell proliferation and DNA synthesis experiments were reported either graphically or in table form showing percent stimulation relative to day 0. For all of these experiments trend analysis of the data was performed with duplicate samples agreeing within 5% of the mean. In most cases significance was determined only following a 200 to 1000 percent increase from day 0.

In addition, statistical analysis was performed for the multiple growth factor cell proliferation assay and the mitogenic response of bone-derived cells to growth factors under controlled conditions assay with the results analysed using an ANOVA with Scheffe's F-test as the post hoc test.

IV. RESULTS

A. Cell Characterization

In order to characterize cell types obtained from explants described above, cells were analyzed morphologically and biochemically with respect to fibroblast and osteoblast phenotypes.

1. Morphology. Minimal differences were noted in the appearances of subconfluent cell cultures (Plates 1,2,3,4). As the cells approached confluence two distinct patterns were observed. Cell populations GF2 and PL7 formed an even monolayer with parallel orientation of fusiform cells in culture flasks (Plates 5,6). In contrast, cell populations BP1 and PL4 readily formed hyperconfluent, multilayered colonies of randomly oriented cells (Plates 7,8).

2. Biochemical assays: Cyclic AMP. Studies were undertaken to measure PTH stimulation af adenyl cyclase activity. In preliminary experiments with BP1 cells the c-AMP response of BP1 cells to PTH was determined without the addition of forskolin. Under these conditions, PTH induced a two-fold increase in c-AMP. In order to optimize conditions for PTH-dependent stimulation of c-AMP, forskolin was added to the cultures with PTH. In low doses forskolin has been shown to potentiate hormone induced adenyl cyclase activity through enhanced guanine nucleotide binding, without significantly increasing basal levels of c-AMP (Seamon and Daly, 1981). The use of forskolin has previously been shown to have a synergistic effect on PTH modulation of c-AMP in osteoblast cultures (Henry et al., 1983; Henry, 1985). Confluent cell cultures of all four cell types

Plate 1

Light microscopic view of a subconfluent cell culture of Normal Adult Human Gingival Fibroblasts (GF2). Magnification x700

Plate 2

Light microscopic view of a subconfluent cell culture of Adult Human Periodontal Ligament/Cementum Cells (PL7). Magnification x700

Plate 3

Light microscopic view of a subconfluent cell culture of Normal Adult Human Bone-Derived Cells (BP1). Magnification x700

Plate 4

Light microscopic view of a subconfluent cell culture of Adult Human Periodontal Ligament/Cementum Cells (PL4). Magnification x700

Plate 5

Light microscopic view of a confluent cell culture of Normal Adult Human Gingival Fibroblasts (GF2). Magnification x700

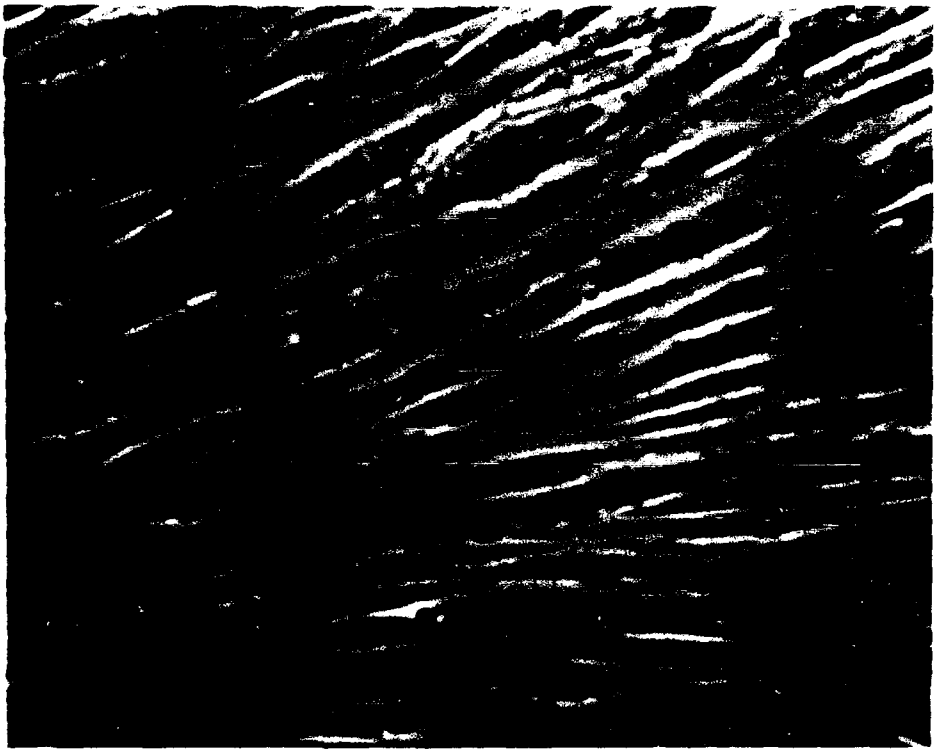


Plate 6

Light microscopic view of a confluent cell culture of Adult Human Periodontal Ligament/Cementum Cells (PL7). Magnification x700

Plate 7

Light microscopic view of a confluent cell culture of Normal Adult Human Bone-Derived Cells (BP1). Magnification x700

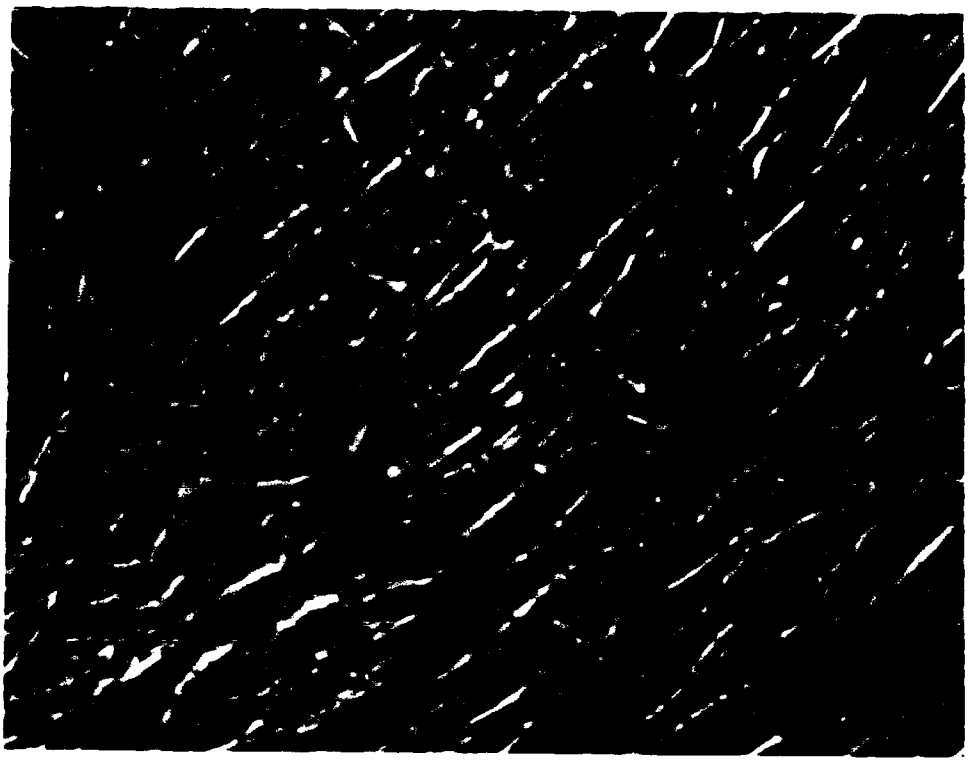
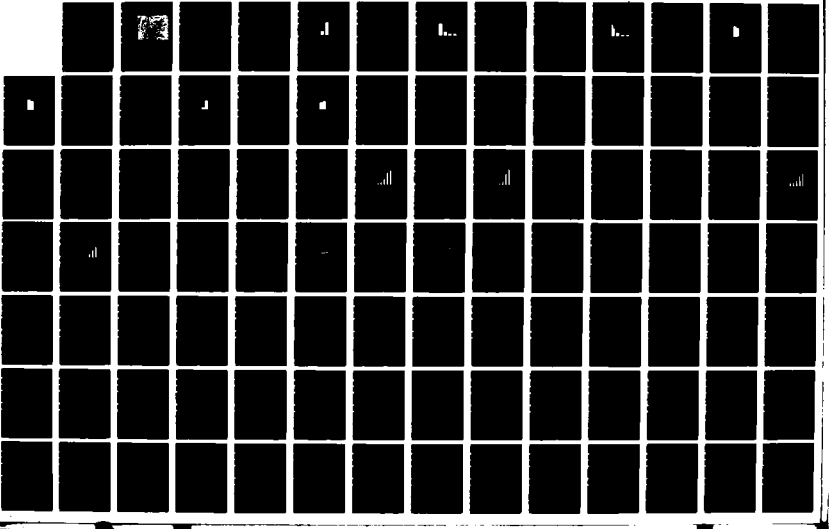


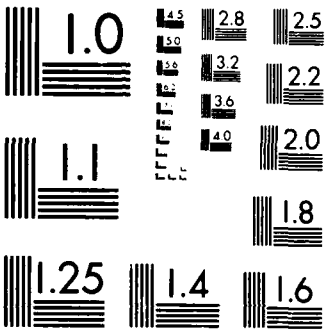
Plate 8

Light microscopic view of a confluent cell culture of Adult Human Periodontal Ligament/Cementum Cells (PL4). Magnification x700

AD-A196 114 INITIAL BIOCHEMICAL CHARACTERIZATION OF CELLS DERIVED FROM HUMAN PERIODON (U) AIR FORCE INST OF TECH WRIGHT-PATTERSON AFB OH J E PICHE' MAY 88 2/3

UNCLASSIFIED AFIT/CI/NR-88-148 F/G 6/1 NL




1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
4.5
5.0
5.6

were assayed for c-AMP production in the presence of forskolin with and without PTH stimulation. Cell numbers ranged from 90,000-100,000 cells/well with viability for all cell types >95% as determined by trypan blue exclusion. All values were normalized to 10^5 cells/well to afford better comparison of cell types. Cell population PL4, derived from the periodontal ligament/cementum, demonstrated the greatest degree of response to PTH with a 19-fold increase in c-AMP levels over nonstimulated cultures (Figure 1) This even exceeded the response of the BP1 bone-derived cells which demonstrated a 6.4-fold increase in c-AMP when stimulated with PTH. PTH stimulated a 1.4-fold increase in c-AMP in the second periodontal ligament/cementum-derived cell type PL7, while the connective tissue-derived fibroblast cell type 2A2 demonstrated no c-AMP response to PTH stimulation.

3. Biochemical assays: Alkaline phosphatase - Parathyroid hormone. Confluent cell cultures were assayed for alkaline phosphatase high endogenous levels of which are an indicator of a bone-derived cell population. Data presented in figure 2 indicate that bone-derived cells BP1 had the highest endogenous level of alkaline phosphatase, which was seven times higher than the level detected in GF2 cells (Figure 2). The periodontal ligament/cementum derived cell type, PL4, had an intermediate basal level of alkaline phosphatase activity, which was twice that observed for GF2 cells (Figure 2). Cell types PL7 and GF2 had the least amount of alkaline phosphatase activity with no significant difference between them (Figure 2).

PTH modulation of alkaline phosphatase activity was also measured since PTH has been shown to diminish alkaline phosphatase activity in

Figure 1

Cyclic AMP response in cells derived from human periodontium. Fifteen minute PTH stimulation. All results were determined from a standard curve prepared with known concentrations of cyclic AMP provided with the Immunonuclear RIA kit. All cyclic AMP samples were run in duplicate. Results are reported as the fold increase relative to unstimulated baseline samples.

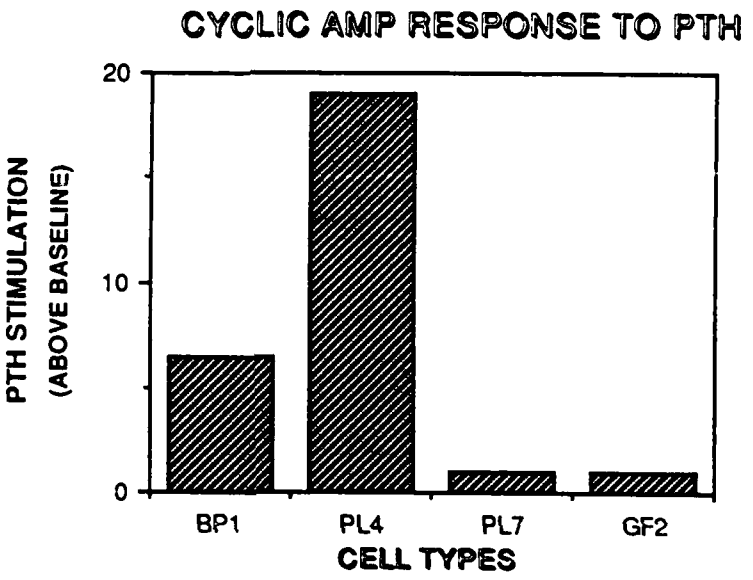
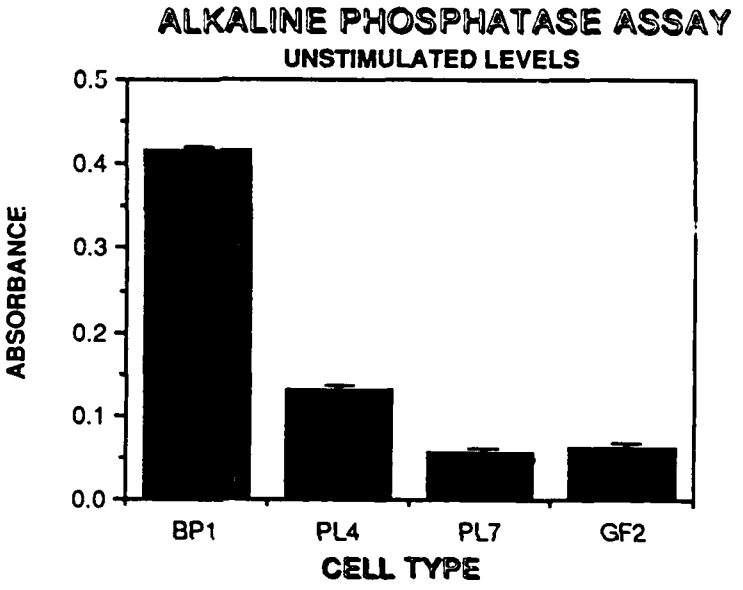

CYCLIC AMP RESPONSE TO PTH
PTH STIMULATION
(ABOVE BASELINE)
20
10
0
BP1
PL4
PL7
GF2
CELL TYPES

Figure 2

Comparison of basal levels of alkaline phosphatase in cells derived from human periodontium. Comparison of unstimulated cells was based on data presented in Figure 2. Significance was determined using Scheffe's F-test. All cell populations had significantly different basal levels of alkaline phosphatase ($p<0.001$) except PL7 and GF2 which showed no significant difference. Error bars indicate the standard error of the mean.


ALKALINE PHOSPHATASE ASSAY
UNSTIMULATED LEVELS
ABSORBANCE
0.5
0.4
0.3
0.2
0.1
0.0
BP1
PL4
PL7
GF2
CELL TYPE

osteoblast cultures (Hekkelman and Moskalewski, 1969; Luben et al., 1976, Thomas and Ramp, 1978; Thomas and Ramp, 1979; Majeska and Rodan, 1982). Initially, all four cell populations were stimulated for 15 minutes with 500 ng/ml of PTH and 1mM isobutylmethylxanthine in HBSS. The exposure of the BP1 cells to PTH induced a 33% decrease in the level of alkaline phosphatase activity which was found to be statistically significant ($p<0.001$). PTH did not significantly affect alkaline phosphatase activity in PL4, PL7, or GF2 cell cultures (Figure 3). Since previous experiments (Luben et al., 1976, Thomas and Ramp, 1978; Majeska and Rodan, 1982) have primarily utilized a 48 hour incubation period with parathyroid hormone to measure the alkaline phosphatase response, the BP1 cells and the PL4 cells were next assayed for alkaline phosphatase using an extended 48 hour exposure to PTH. When the PL4 cells were exposed to 100 ng/ml of PTH without IBMX for 48 hours a statistically significant ($p<0.001$) 38% decrease in alkaline phosphatase was observed (Figure 4). This was similar to the statistically significant 23% decrease which was seen when the BP1 cells were exposed to 200 ng/ml of PTH for 48 hours (Figure 5) and is consistent with the results found in the .previously referenced articles. Therefore, the BP1 cells were able to respond to a short 15 minute exposure to parathyroid hormone in the presence of IBMX while the PL4 cells required a significantly longer 48 hour exposure to parathyroid hormone before a significant decrease in alkaline phosphatase levels was seen.

4. Biochemical assays: 1,25 DihydroxyVitamin D. The response of the BP1 and PL4 cell populations to 1,25 Dihydroxyvitamin D was also measured since this biochemical marker has been shown to cause an increase in basal alkaline phosphatase in bone cells but not fibroblasts (Beresford

Figure 3

Alkaline phosphatase activity in cell derived from human periodontium. Fifteen minute PTH stimulation. Alkaline phosphatase activity was measured by absorbance at 410 nm as described in the text. Samples were run in quadruplicate. Significance was determined using Scheffe's F-test. Only cell population BP1 demonstrated a significant change ($p<0.001$). The remaining cell populations did not have a significant change in alkaline phosphatase levels when exposed to PTH. Error bars indicate the standard error of the mean.

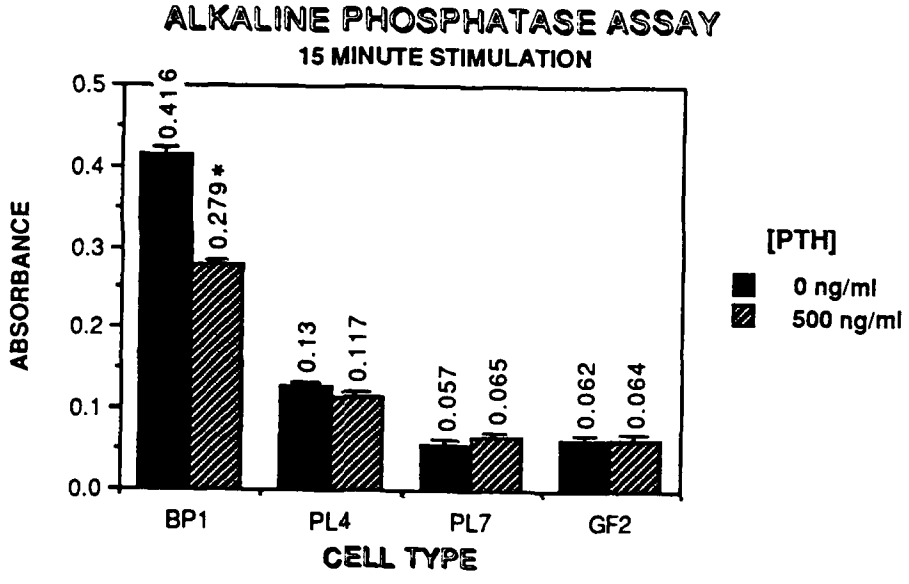

ALKALINE PHOSPHATASE ASSAY
15 MINUTE STIMULATION
ABSORBANCE
0.5
0.4
0.3
0.2
0.1
0.0
0.416
0.279*
0.13
0.117
0.057
0.065
0.062
0.064
BP1
PL4
PL7
GF2
CELL TYPE
[PTH]
0 ng/ml
500 ng/ml

Figure 4

Alkaline phosphatase activity in periodontal ligament cells (PL4) derived from human periodontium. The effect of a 48 hour incubation using multiple concentrations of PTH was examined for human periodontal ligament/cementum cells. Alkaline phosphatase was measured by absorbance at 410 nm. Samples were run in quadruplicate. Significance was determined using Scheffe's F-test. * indicates significance at the $p<0.05$ level. Error bars indicate the standard error of the mean.

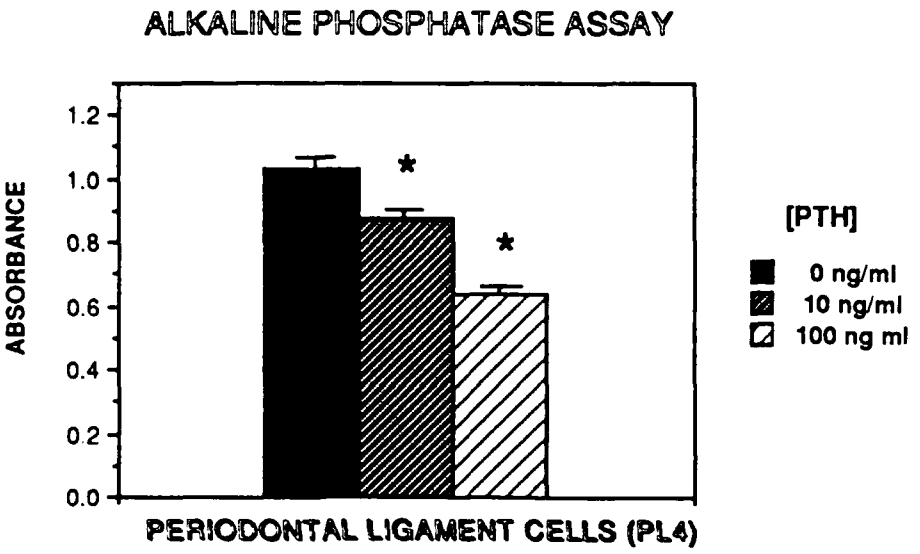
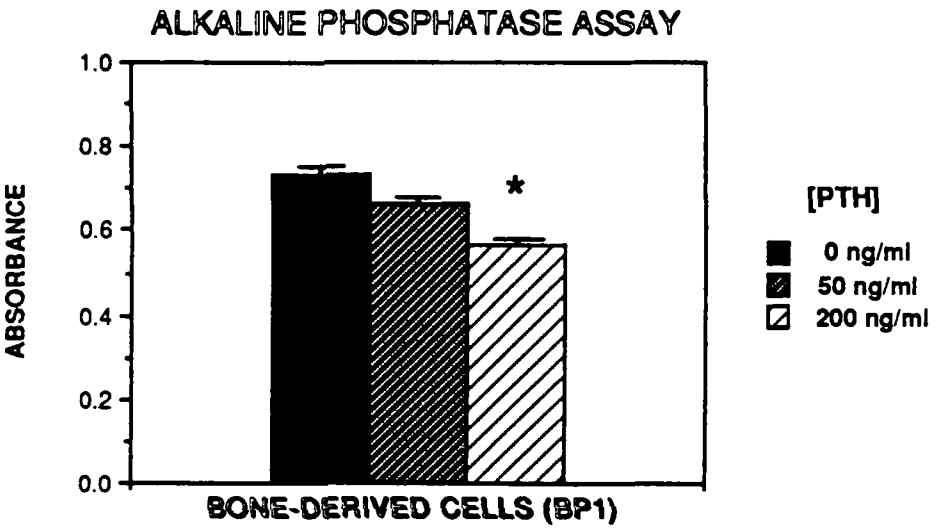

ALKALINE PHOSPHATASE ASSAY
ABSORBANCE
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
PERIODONTAL LIGAMENT CELLS (PL4)
[PTH]
0 ng/ml
10 ng/ml
100 ng ml

Figure 5

Alkaline phosphatase activity in normal human bone-derived cells. The effect of a 48 hour incubation using multiple concentrations of PTH was examined for normal human bone-derived cells. Alkaline phosphatase was measured by absorbance at 410 nm. Samples were run in quadruplicate. Significance was determined using Scheffe's F-test. * indicates significance at the $p<0.05$ level. Error bars indicate the standard error of the mean.


ALKALINE PHOSPHATASE ASSAY
ABSORBANCE
1.0
0.8
0.6
0.4
0.2
0.0
*
BONE-DERIVED CELLS (BP1)
[PTH]
0 ng/ml
50 ng/ml
200 ng/ml

et al., 1986). It was hoped that this assay would help to differentiate the bone-derived cell population (BP1) and the periodontal ligament/cementum cell population (PL4). As seen in figure 6, when physiologic concentrations of 1,25 dihydroxyvitamin D were added to the culture medium for 48 hours a statistically significant 450% increase in alkaline phosphatase was obtained with the periodontal ligament/cementum (PL4) cells (Figure 6). Although the relative increase was not as large for the periodontal ligament/cementum cells (PL4), a statistically significant 60% increase was also obtained when the BP1 cell population was incubated with the same concentrations of 1,25 DihydroxyVitamin D (Figure 7).

B. Response to Growth Factors

Since bone cells respond differently than fibroblasts to hormonal control, it was felt that these cell populations would also respond differently to locally generated paracrine growth factors. Initially, all cell populations were tested for proliferative response to platelet-derived growth factor. Basal media, DMEM, was supplemented with platelet poor plasma since platelet-poor plasma contains essential nutrients including lipids, is minimally stimulatory for fibroblasts and enhances the response of fibroblasts to PDGF (Pledger et al., 1977; Ross and Vogel, 1978; Antoniades and Owen, 1982; Pledger et al., 1982; Antoniades and Owen, 1984). In addition, the concentrations of platelet-poor plasma used contain very little PDGF (Cochran et al., 1983). Bone-derived cells proliferated in 4% platelet-poor plasma (Figure 8) so that the cell number increased five-fold over nine days. Low concentrations of PDGF (0.6 ng/ml)

Figure 6

Alkaline phosphatase activity in periodontal ligament cells (PL4) derived from human periodontium. The effect of a 48 hour incubation using multiple concentrations of 1,25 dihydroxyvitamin D3 was examined for human periodontal ligament/cementum cells. Alkaline phosphatase was measured by absorbance at 410 nm. Samples were run in quadruplicate. Significance was determined using Scheffe's F-test. * indicates significance at the $p<0.001$ level. Error bars indicate the standard error of the mean.

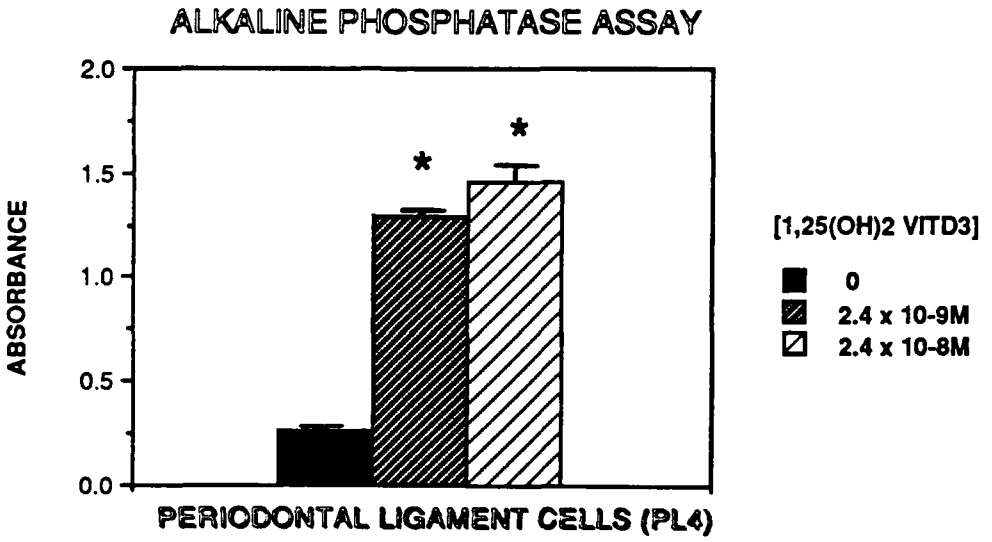

ALKALINE PHOSPHATASE ASSAY
2.0
1.5
1.0
0.5
0.0
ABSORBANCE
*
*
PERIODONTAL LIGAMENT CELLS (PL4)
[1,25(OH)2 VITD3]
0
2.4 x 10-9M
2.4 x 10-8M

Figure 7

Alkaline phosphatase activity in normal human bone-derived cells. The effect of a 48 hour incubation using multiple concentrations of 1,25 dihydroxyvitamin D3 was examined for human bone-derived cells. Alkaline phosphatase was measured by absorbance at 410 nm. Samples were run in quadruplicate. Significance was determined using Scheffe's F-test. * indicates significance at the $p<.001$ level. Error bars indicate the standard error of the mean.

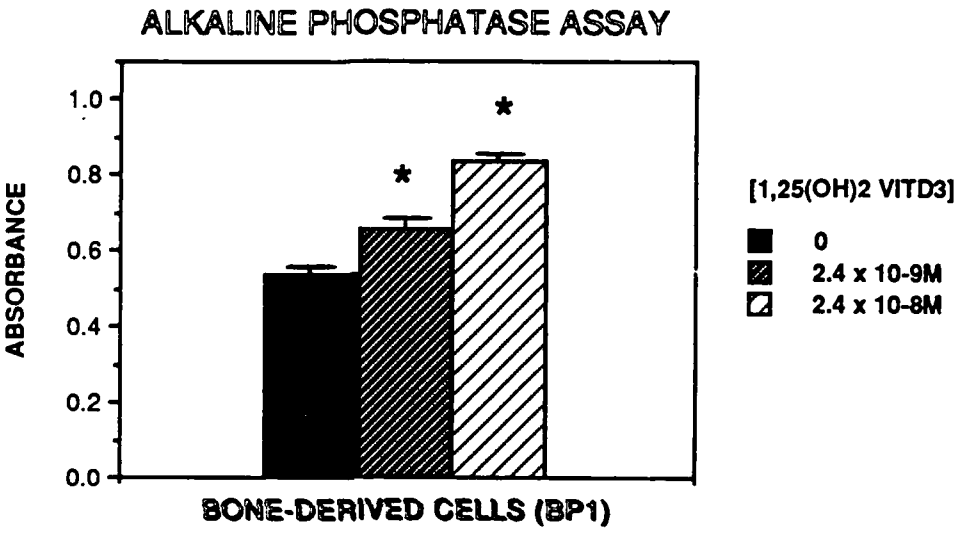
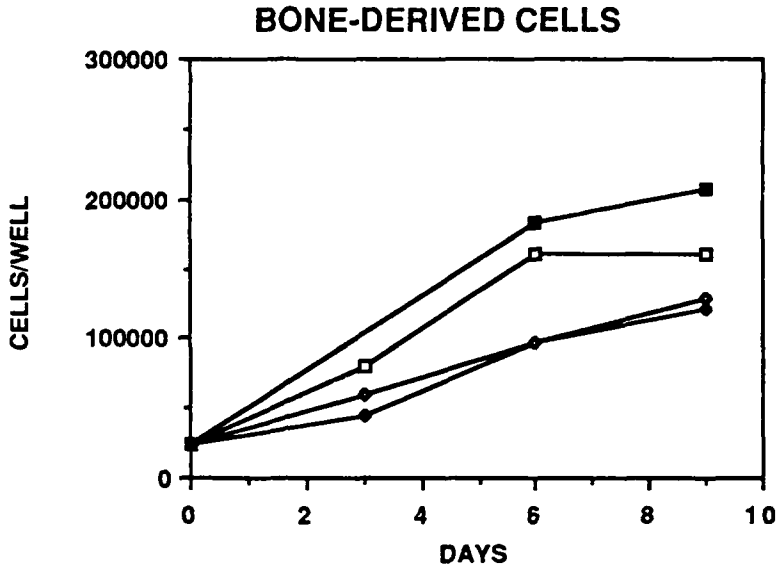

ALKALINE PHOSPHATASE ASSAY
ABSORBANCE
1.0
0.8
0.6
0.4
0.2
0.0
*
*
BONE-DERIVED CELLS (BP1)
[1,25(OH)2 VITD3]
0
2.4 x 10-9M
2.4 x 10-8M

Figure 8

Proliferation of bone-derived cells (BP1) in response to PDGF. Bone-derived cells were plated at subconfluence in 10% FBS, incubated overnight and changed to DMEM supplemented with 4% platelet-poor plasma (PPP) and 0, ◆ ; 0.6, ◇ ; and 6.0 ng/ml PDGF, □ ; or DMEM supplemented with 10% FBS, ■ . Cells were counted on days 0, 3, 6 and 9.

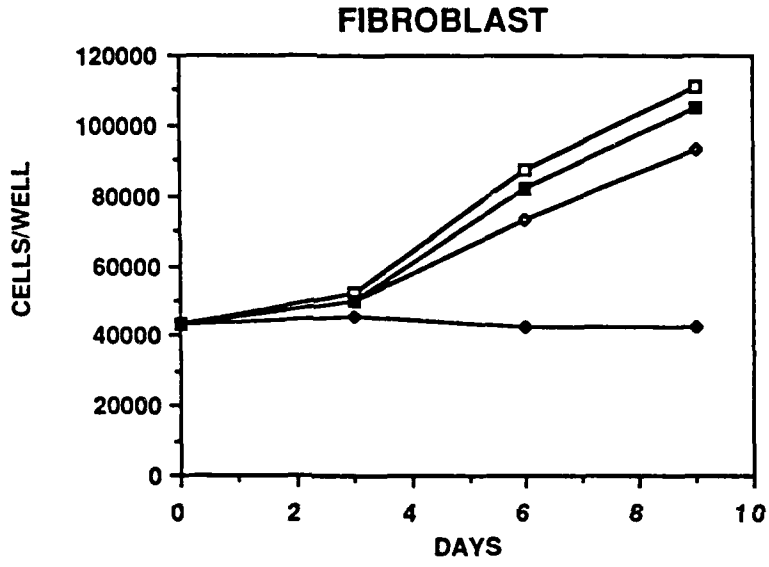

BONE-DERIVED CELLS
300000
200000
100000
0
CELLS/WELL
0
2
4
6
8
10
DAYS

were not stimulatory compared to cells in control medium. At higher concentrations, 6.0 ng/ml of PDGF stimulated a 33% percent greater increase in cell number over nine days when compared to cells in 4% PPP alone. This was still less than the 71% increase observed in cells incubated in 10% FBS. Ten-percent FBS was chosen for comparison because it provides an enriched supplement of growth factors and nutrients which has been found to be highly stimulatory for cells of mesenchymal origin. In contrast to the bone-derived cells, fibroblasts did not proliferate in low concentrations of platelet-poor plasma (Figure 9) as has been previously demonstrated (Pledger et al., 1977; Clemmons and Van Wyk, 1981; Graves et al., 1983). Low concentrations of PDGF (0.6 ng/ml) were stimulatory while 6 ng/ml PDGF stimulated cellular proliferation to the same extent as cells incubated in 10% FBS. The response observed for fibroblasts is consistent with previous reports that the mitogenic effect of PDGF for other cells of mesenchymal origin, such as smooth muscle cells, fibroblasts, and glial cells, is maximized between 2-5 ng/ml (Ross et al., 1974; Westermark and Wasteson, 1976; Pledger et al., 1977; Antoniades and Owen, 1984). The above results indicate that normal human fibroblasts are more responsive to physiologic concentrations of PDGF than the bone-derived cells.

When the proliferative response of the periodontal ligament/cementum cells is examined two patterns emerged; with one pattern similar to the bone-derived cells and one pattern similar to the fibroblasts. The PL4 cells responded similar to the bone-derived cells in that they also proliferated in 4% platelet-poor plasma, demonstrating a 3.4-fold increase in nine days. Low concentrations of PDGF (0.6 ng/ml) were again nonstimulatory, while the higher concentration of PDGF (6.0 ng/ml) produced

Figure 9

Proliferation of fibroblasts (GF2) in response to PDGF. Fibroblasts were plated at subconfluence in 10% FBS, incubated overnight and changed to DMEM supplemented with 4% platelet-poor plasma (PPP) and 0, ◆ ; 0.6, ◇ ; and 6.0 ng/ml PDGF, □ ; or DMEM supplemented with 10% FBS, ■ . Cells were counted on days 0, 3, 6 and 9.


FIBROBLAST
120000
100000
80000
60000
40000
20000
0
CELLS/WELL
0
2
4
6
8
10
DAYS

a 15% increase in cell number relative to the 4% PPP negative control (Figure 10). In contrast, the PL7 cell population responded in a manner similar to the fibroblasts. No proliferation of cells was seen in medium supplemented with only platelet-poor plasma, while addition of 0.6 ng/ml to the culture medium significantly stimulated cellular proliferation. Similar to the fibroblasts, maximum cellular proliferation of the PL7 cells was obtained with 6.0 ng/ml of PDGF which essentially equalled the proliferation seen in cells incubated with 10% FBS (Figure 11). These results indicate that the PL7 periodontal ligament/cementum cell population is more responsive to low concentrations of PDGF than the PL4 periodontal ligament/cementum cells.

Since bone-derived cells and the PL4 periodontal ligament/cementum cells were apparently able to utilize the growth factors present in platelet-poor plasma and proliferate, the response of these cells to platelet-poor plasma was examined (Table 1,2). With regard to the bone-derived cells, platelet-poor plasma stimulated a dose dependent increase in the number of bone cells, with 10% platelet-poor plasma demonstrating a 723% increase over six days, which was still less stimulatory than 10% FBS (Table 1). Similarly, 10% platelet-poor plasma stimulated a 273% increase in the number of PL4 cells over a six day period which was also significantly less stimulatory than the 10% FBS (Table 2). These results were consistent in multiple assays (data not shown).

In order to further explore this aspect of cellular response, autoradiographic studies were undertaken to determine the actual percentage of cells responding to PDGF and PPP. When the response of bone-derived and fibroblast cell populations were tested in incubation medium containing 3%

Figure 10

Proliferation of periodontal ligament/cementum cells (PL4) in response to PDGF. PL4 cells were plated at subconfluence in 10% FBS, incubated overnight and changed to DMEM supplemented with 4% platelet-poor plasma (PPP) and 0, ◆ ; 0.6, ◇ ; and 6.0 ng/ml PDGF, □ ; or DMEM supplemented with 10% FBS, ■ . Cells were counted on days 0, 3, 6 and 9.

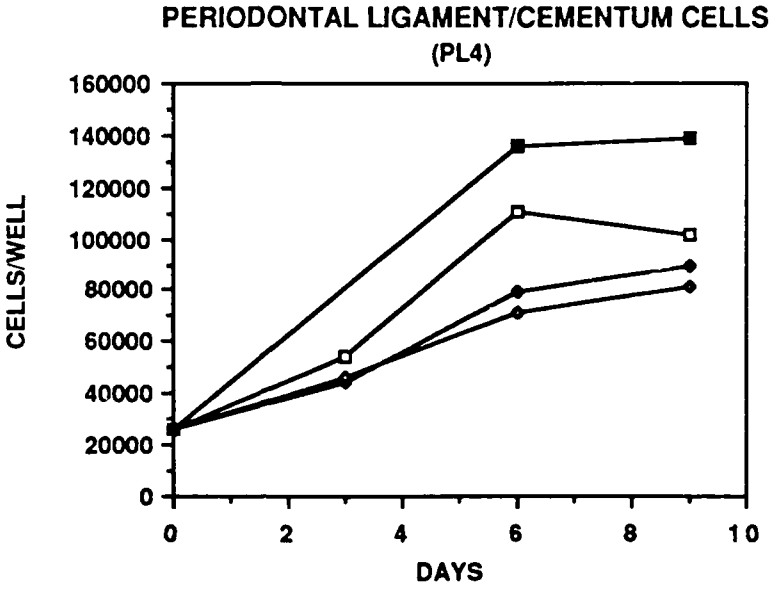

PERIODONTAL LIGAMENT/CEMENTUM CELLS
(PL4)
CELLS/WELL
160000
140000
120000
100000
80000
60000
40000
20000
0
0
2
4
6
8
10
DAYS

Figure 11

Proliferation of periodontal ligament/cementum cells (PL7) in response to PDGF. PL7 cells were plated at subconfluence in 10% FBS, incubated overnight and changed to DMEM supplemented with 4% platelet-poor plasma (PPP) and 0, ◆ ; 0.6, ◇ ; and 6.0 ng/ml PDGF, □ ; or DMEM supplemented with 10% FBS, ■ . Cells were counted on days 0, 3, 6 and 9.

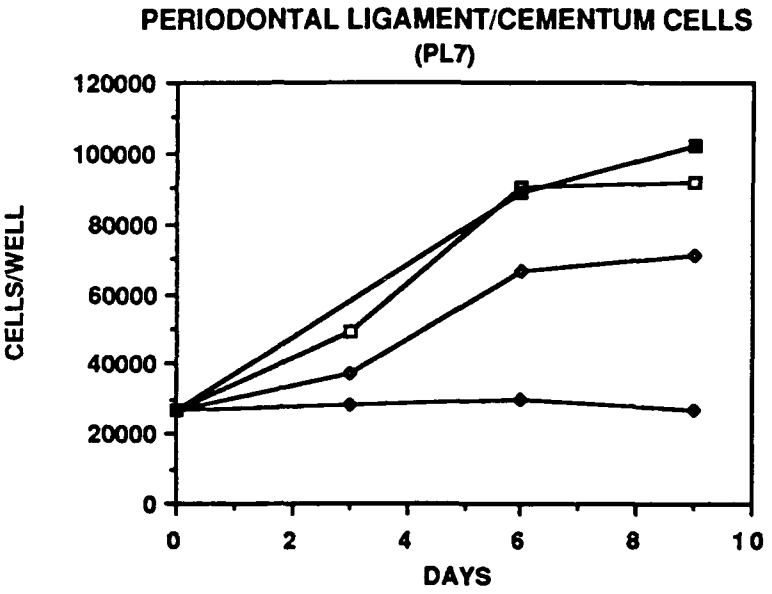

PERIODONTAL LIGAMENT/CEMENTUM CELLS
(PL7)
CELLS/WELL
120000
100000
80000
60000
40000
20000
0
0
2
4
6
8
10
DAYS

Table 1

GROWTH OF NORMAL HUMAN BONE-DERIVED CELLS
IN PLATELET-POOR PLASMA

Medium	Cell Number	Percent change in cell number over 6 days
DMEM	4,333	-50
3%PPP/DMEM	27,166	+200
10%PPP/DMEM	72,500	+723
10%FBS/DMEM	146,500	+1565

Day 0 cell counts were 8,800 cells/well. Following incubation with the indicated culture media the cell number was determined after 6 days. Each number represents the mean value fro duplicate wells. Duplicates agreed within 10% of the mean.

Table 2

GROWTH OF PERIODONTAL LIGAMENT/CEMENTUM CELLS (PL4) IN PLATELET-POOR PLASMA

Medium	Cell Number	Percent change in cell number over 6 days
DMEM	3,500	-26
3%PPP/DMEM	46,000	+240
10%PPP/DMEM	51,500	+281
10%FBS/DMEM	100.000	+641

Day 0 cell counts were 13,500 cells/well. Following incubation with the indicated culture media the cell number was determined after 6 days. Each number represents the mean value for duplicate wells. Duplicates agreed within 10% of the mean.

platelet-poor plasma and 10 ng/ml of PDGF, approximately 50% of the nuclei of both cell types were labelled. This was slightly less than the percentage of cells responding to 10% FBS. In addition, a dose dependent response to decreasing concentrations of PDGF was seen. While 10 ng/ml of PDGF caused a six-fold increase in the number of bone-derived cells and a 42-fold increase in the number of fibroblasts relative to the 3% platelet-poor plasma negative control, 2.5 ng/ml of PDGF induced a three-fold (bone) and an eight-fold (fibroblast) increase in DNA synthesis. In addition, 0.5 ng/ml of PDGF was stimulatory for fibroblasts but not bone-derived cells (Figures 12,13). Another aspect of this data is that pre-incubation with insulin and transferrin reduced the response of both cell types to PDGF and 10% FBS when compared to the response in cells depleted in platelet-poor plasma.

Probably the major difference in the proliferative response of the bone-derived cells and the fibroblasts as measured by autoradiography is reflected in the negative control (Table 3). When cells were incubated in 3% platelet-poor plasma only 1.2% of the normal human fibroblast nuclei were labelled, in contrast to bone-derived cells in which 8.8% of the nuclei were labelled. This indicates that a considerably larger number of bone-derived cells are synthesizing DNA under conditions in which fibroblasts are quiescent. Experiments with cells tested under more defined conditions provide further insight into this response. When incubated in insulin and transferin in lieu of platelet-poor plasma, all normal human fibroblasts and bone-derived cells were virtually quiescent, with 0.9% and 0.6% respectively having labelled nuclei. This would suggest that there are factors in platelet-poor plasma other than insulin,

Figure 12

PDGF stimulates an elevated percentage of normal adult human bone-derived cells to synthesize DNA. Bone-derived cells were plated at 10,000 cells/well and grown to confluence. Solid bars represent cells depleted and assayed in DMEM supplemented with 3% platelet-poor plasma; hatched bars represent cells depleted and assayed in DMEM supplemented with insulin (5 Ug/ml) and transferrin (5Ug/ml). Assay medium was supplemented with 0,(A); 0.5,(B); 2.5, (C); and 10 ng/ml PDGF; or in DMEM supplemented with 10% FBS,(E). All concentrations were run in quadruplicate with mean $\pm$ standard error of the mean calculated.

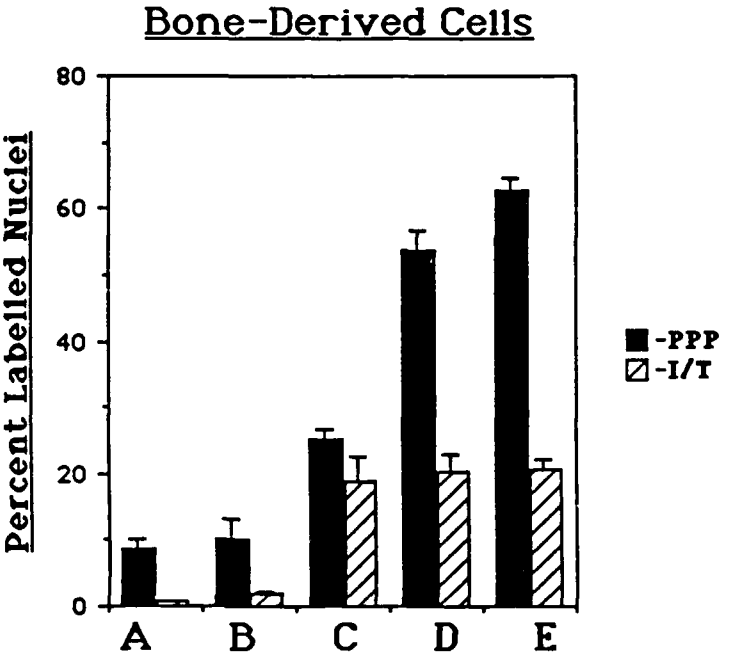

Bone-Derived Cells
Percent Labelled Nuclei
80
60
40
20
0
A
B
C
D
E
-PPP
-I/T

Figure 13

PDGF stimulates an elevated percentage of normal adult human gingival fibroblasts to synthesize DNA. Fibroblasts were plated at 10,000 cells/well and grown to confluence. Solid bars represent cells depleted and assayed in DMEM supplemented with 3% platelet-poor plasma; hatched bars represent cells depleted and assayed in DMEM supplemented with insulin (5 Ug/ml) and transferrin (5Ug/ml). Assay medium was supplemented with 0,(A); 0.5,(B); 2.5, (C); and 10 ng/ml PDGF; or in DMEM supplemented with 10% FBS,(E). All concentrations were run in quadruplicate with mean $\pm$ standard error of the mean calculated.

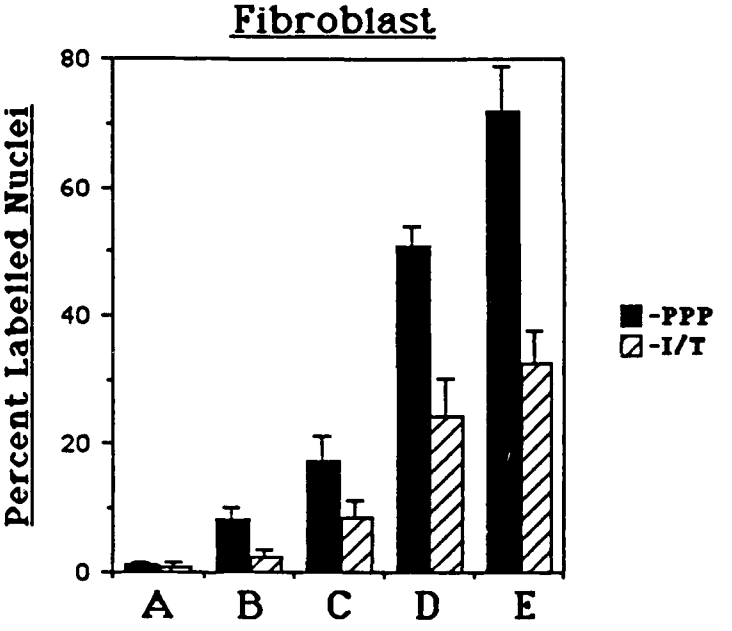

Fibroblast
Percent Labelled Nuclei
80
60
40
20
0
A
B
C
D
E
-PPP
-I/T

Table 3

MITOGENIC RESPONSE OF BONE-DERIVED CELLS AND FIBROBLASTS TO PLATELET-POOR PLASMA:

AUTORADIOGRAPHIC ANALYSIS

BONE-DERIVED CELLS

Medium	Percent of Labelled Nuclei Mean ± S.E.M.
Insulin/ Transferrin	0.5 ± 0.18
PPP	8.8 ± 0.85
FBS	62.0 ± 1.45

FIBROBLASTS

Medium	Percent of Labelled Nuclei Mean ± S.E.M.
Insulin/ Transferrin	0.9 ± 0.44
PPP	1.2 ± 0.30
FBS	71.7 ± 5.81

Bone-derived cells and fibroblasts were plated at 10,000 cells/well and grown to confluence. Cells were incubated in DMEM supplemented with insulin (5Ug/ml) and transferrin (5Ug/ml), 3% PPP, or 10% FBS for 72 hours. The culture medium was changed and DNA synthesis measured by autoradiography. Each number represents the mean of triplicate wells ± standard error of the mean. Triplicates agreed within 10% of the mean.

insulin-like growth factors, or transferrin which support DNA synthesis in bone-derived cells but not fibroblasts.

When the ability of the periodontal ligament/cementum cells to synthesize DNA upon PDGF stimulation was examined, an interesting parallel to the response of fibroblasts and bone-derived cells was again seen (Table 4, Figures 14,15). Both periodontal ligament/cementum cell populations (PL4, PL7) showed a dose dependent response to platelet-derived growth factor when tested in medium supplemented with platelet-poor plasma or insulin and transferrin (Figures 14,15). However, for both cell types, the percentage of cells responding was considerably lower than the percent of fibroblasts and bone-derived cells responding, although the relative changes were similar. When the response to the negative controls was examined the results were virtually identical to those seen with the fibroblast and bone-derived cells. The PL4 cell population again demonstrated a response similar to that seen in the bone-derived cells with 5.7% of the nuclei labeled when quiesced in 3% PPP and only 0.7% of the nuclei labeled when quiesced in medium supplemented with insulin and transferrin (Table 4). In contrast, the PL7 cell population demonstrated virtual quiescence in 3% PPP and insulin and transferrin with only 1.2% and 0.9% of the nuclei labelled (Table 4). This response was identical to that seen in the fibroblast cell population.

The next series of experiments examined the effects when PDGF, EGF, and TGF-B are added individually to the bone-derived and fibroblast cell populations in varying concentrations. Due to the fact that the periodontal ligament/cementum cells had behaved in essentially an identical manner to the bone-derived cells and fibroblasts regarding their response

Table 4
MITOGENIC RESPONSE OF PERIODONTAL LIGAMENT/
CEMENTUM CELLS TO PLATELET-POOR PLASMA:
AUTORADIOGRAPHIC ANALYSIS

PERIODONTAL LIGAMENT/CEMENTUM CELLS

PL4

Medium	Percent of Labelled Nuclei Mean ± S.E.M.
Insulin/ Transferrin	0.7 ± 0.10
PPP	5.7 ± 0.65
FBS	23.0 ± 1.10

PL7

Medium	Percent of Labelled Nuclei Mean ± S.E.M.
Insulin/ Transferrin	0.8 ± 0.30
PPP	0.8 ± 0.25
FBS	18.0 ± 2.55

Periodontal ligament/cementum cells were plated at 10,000 cells/well and grown to confluence. Cells were incubated in DMEM supplemented with insulin (5Ug/ml) and transferrin (5Ug/ml), 3% PPP, or 10% FBS for 72 hours. The culture medium was changed and DNA synthesis was measured by autoradiography. Each number represents the mean of triplicate wells ± standard error of the mean. Triplicates agreed within 10% of the mean.

Figure 14

PDGF stimulates an elevated percentage of adult human periodontal ligament/cementum cells (PL4) to synthesize DNA. PL4 cells were plated at 10,000 cells/well and grown to confluence. Solid bars represent cells depleted and assayed in DMEM supplemented with 3% platelet-poor plasma; hatched bars represent cells depleted and assayed in DMEM supplemented with insulin (5 Ug/ml) and transferrin (5Ug/ml). Assay medium was supplemented with 0,(A); 0.5,(B); 2.5, (C); and 10 ng/ml PDGF; or in DMEM supplemented with 10% FBS,(E). All concentrations were run in quadruplicate with mean $\pm$ standard error of the mean calculated.

PERIODONTAL LIGAMENT/CEMENTUM

PL4

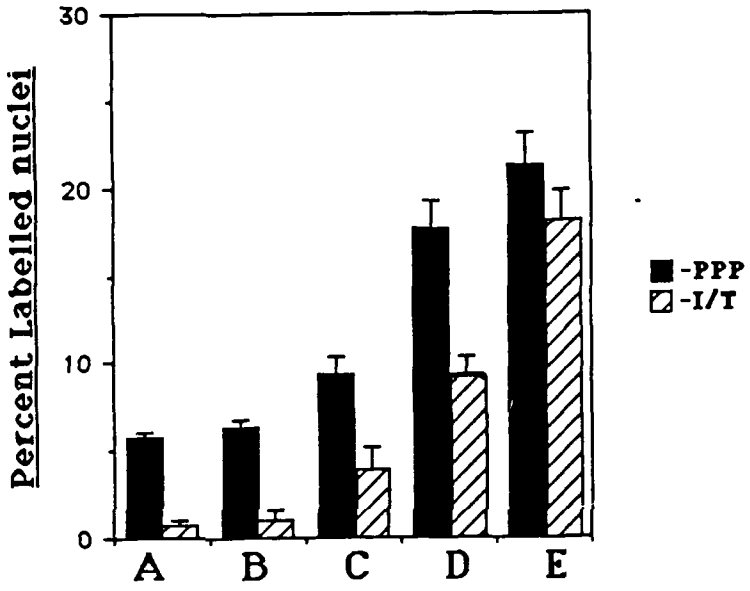
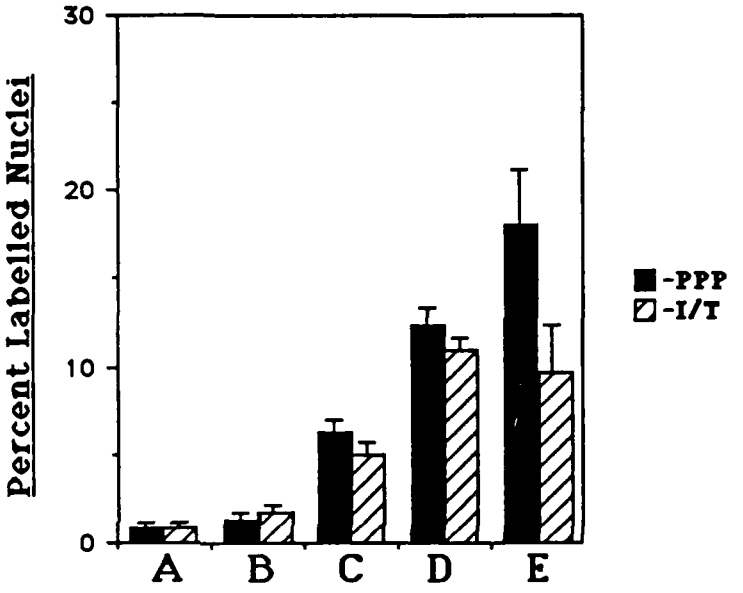


Figure 15

PDGF stimulates an elevated percentage of adult human peridontal ligament/cementum cells (PL7) to synthesize DNA. PL7 cells were plated at 10,000 cells/well and grown to confluence. Solid bars represent cells depleted and assayed in DMEM supplemented with 3% platelet-poor plasma; hatched bars represent cells depleted and assayed in DMEM supplemented with insulin (5 Ug/ml) and transferrin (5Ug/ml). Assay medium was supplemented with 0,(A); 0.5,(B); 2.5, (C); and 10 ng/ml PDGF; or in DMEM supplemented with 10% FBS,(E). All concentrations were run in quadruplicate with mean $\pm$ standard error of the mean calculated.

PERIODONTAL LIGAMENT/CEMENTUM

(PL7)



to growth factor stimulation, and because of the high cost involved in this series of experiments, only normal human bone-derived and fibroblast cell populations were utilized in the remaining experiments. When varying concentrations of the growth factors were added singly to the culture medium supplemented with 2% platelet-poor plasma it was found that bone-derived cells did not respond to low concentrations of TGF-B (0.2 and 1.5 ng/ml), but did demonstrate a 28% increase in cellular proliferation above the negative control when incubated with 7.0 ng/ml of TGF-beta. Incubation of the bone-derived cells with 0.5 and 2.5 ng/ml of EGF also induced a 29% and 44% increase in cell number above the negative control. PDGF (6.0 ng/ml) was found to stimulate the greatest increase in cellular proliferation in the bone-derived cells, which again did not equal the proliferative response seen in cells incubated in 10% FBS (Table 5, Figure 16). When tested for stimulation of fibroblasts TGF-B did not enhance proliferation and EGF was, at most, weakly mitogenic. However, fibroblasts were highly responsive to 6.0 ng/ml of PDGF which was as mitogenic as 10% FBS (Table 6, Figure 17).

Since cells responding in a wound healing environment are exposed to multiple growth factors, the response of normal human fibroblasts and bone-derived cells to combinations of growth factors was tested (Tables 7,8; Figures 18,19). The concentrations of growth factors used in this series of experiments were based on concentrations previously reported to be stimulatory for a variety of cell types of mesenchymal origin (Ross and Vogel, 1978; Antoniades et al., 1979; Heldin et al., 1979; Roberts et al., 1981; Assoian et al., 1983; Wrana et al., 1986; Centrella et al., 1987). In addition, the concentrations of TGF-B and EGF-like activity chosen are

Table 5

PROLIFERATION IN RESPONSE TO GROWTH FACTORS

BONE-DERIVED CELLS

Sample	Cell Number	% Increase over control	% Increase over 7 days
Negative Control	50,500		106
10% NBS	112,000	122	357
0.2ng/ml B-TGF	50,500	0	106
1.5ng/ml B-TGF	53,000	5	116
7.0ng/ml B-TGF	64,000	27	161
0.5ng/ml EGF	65,000	29	165
2.5ng/ml EGF	72,000	43	194
6.0ng/ml PDGF	88,500	75	261

Day 0 cell counts were 24,500 cells/well. Cells were incubated in DMEM supplemented with 10% FBS or 3% PPP with control buffer or growth factor. Media was changed on day four and cells counted on day seven. Each number represents the mean of duplicate samples. Duplicates agreed within 5% of the mean.

Figure 16

Proliferation of bone-derived cells in response to PDGF, EGF, or TGF-beta. Bone-derived cells were plated at subconfluence in 10% FBS and incubated overnight. Cells were assayed in 10% FBS, ■ ; 4% PPP, ◆ ; or 4% PPP supplemented with: 0.2 ng/ml TGF-beta, × ; 1.5 ng/ml TGF-beta, ◇ ; 7.0 ng/ml TGF-beta, + ; 0.5 ng/ml EGF, △ ; 2.5 ng/ml EGF, ▲ ; or 6.0 ng/ml PDGF, □ . Cells were counted on days 4 and 7.

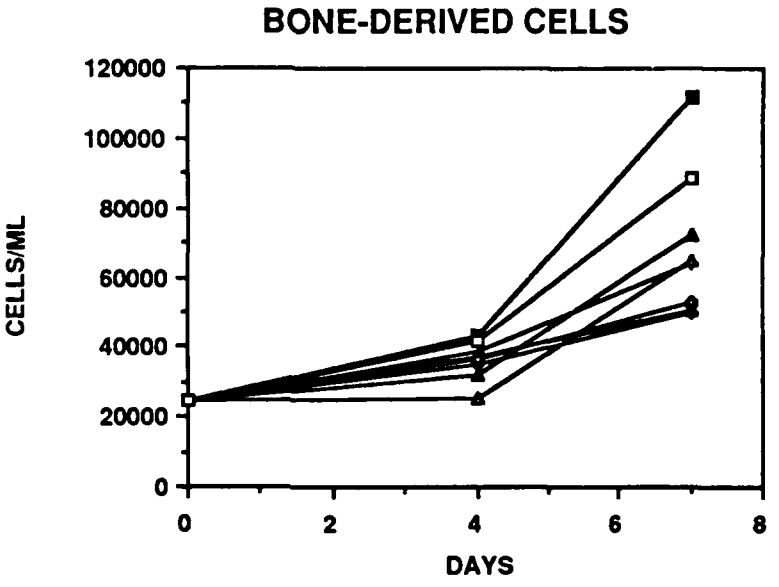

BONE-DERIVED CELLS
120000
100000
80000
60000
40000
20000
0
CELLS/ML
0
2
4
6
8
DAYS

Table 6

PROLIFERATION IN RESPONSE TO GROWTH FACTORS

FIBROBLASTS

Sample	Cell Number	% Increase over control	% Increase over 7 days
Negative Control	17,500		-14
10% NBS	62,500	257	205
0.2ng/ml B-TGF	22,000	26	7
1.5ng/ml B-TGF	19,500	11	-5
7.0ng/ml B-TGF	21,000	20	2
0.5ng/ml EGF	18,500	6	-9
2.5ng/ml EGF	26,000	49	27
6.0ng/ml PDGF	63,500	263	210

Day 0 cell counts were 20,500 cells/well. Cells were incubated in DMEM supplemented with 10% Fetal Bovine Serum or 3% Platelet-Poor Plasma with control buffer or growth factor. Media was changed on day four and cells counted on day seven. Each number represents the mean of duplicate samples. Duplicates agreed within 5% of the mean.

Figure 17

Proliferation of normal human fibroblasts in response to PDGF, EGF, or TGF-beta. Bone-derived cells were plated at subconfluence in 10% FBS and incubated overnight. Cells were assayed in 10% FBS, ■ ; 4% PPP, ◆ ; or 4% PPP supplemented with: 0.2 ng/ml TGF-beta, ✕ ; 1.5 ng/ml TGF-beta, ◇ ; 7.0 ng/ml TGF-beta, + ; 0.5 ng/ml EGF, △ ; 2.5 ng/ml EGF, ▲ ; or 6.0 ng/ml PDGF, □ . Cells were counted on days 4 and 7.

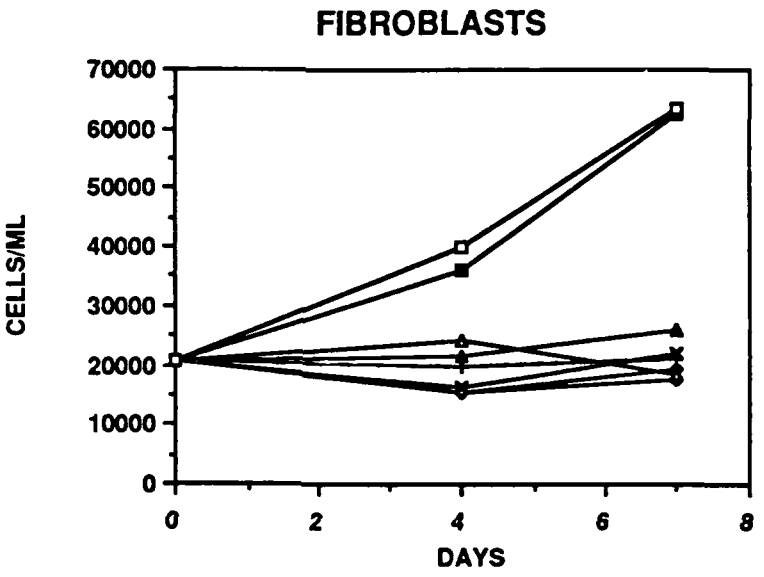

FIBROBLASTS
70000
60000
50000
40000
30000
20000
10000
0
CELLS/ML
0
2
4
6
8
DAYS

Table 7

PROLIFERATIVE RESPONSE OF BONE-DERIVED CELLS TO MULTIPLE GROWTH FACTORS

Sample	Cell Number Mean ± S.E.M.	Significance (*) Relative To: 2% PPP	10% FBS
Negative Control (2% PPP)	39,667 ± 1,201	NS	*
TGF-B + EGF	54,333 ± 1,453	NS	*
TGF-B + PDGF	73,333 ± 1,764	*	*
PDGF + EGF	75,667 ± 1,202	*	*
TGF-B + PDGF + EGF	97,000 ± 3,000	*	NS
10% FBS	101,333 ± 4,055	*	NS

Day 0 cell counts were 13,000 cells/well. Cells were incubated in 10% FBS, 2% PPP or 2% PPP supplemented with the following growth factors in combination: 7.0 ng/ml TGF-B, 2.5 ng/ml PDGF, 2.5 ng/ml EGF. Each value represents the mean of triplicate samples ± the standard error of the mean obtained on day 9 in Figure 18. Statistical significance was determined between groups using Scheffe's F-Test at the $p < 0.01$ level. Values that are significantly different are denoted by (*). NS indicates no statistical significance.

Table 8

PROLIFERATIVE RESPONSE OF FIBROBLASTS TO MULTIPLE GROWTH FACTORS

Sample	Cell Number Mean ± S.E.M.	Significance (*) Relative To: 2% PPP	10% FBS
Negative Control (2% PPP)	17,000 ± 577	NS	*
TGF-B + EGF	20,000 ± 577	NS	*
TGF-B + PDGF	48,333 ± 1,333	*	NS
PDGF + EGF	48,333 ± 882	*	NS
TGF-B + PDGF + EGF	52,667 ± 1,333	*	NS
10% FBS	51,667 ± 882	*	NS

Day 0 cell counts were 18,000 cells/well. Cells were incubated in 10% FBS, 2% PPP or 2% PPP supplemented with the following growth factors in combination: 7.0 ng/ml TGF-B, 2.5 ng/ml PDGF, 2.5 ng/ml EGF. Each value represents the mean of triplicate samples ± the standard error of the mean obtained on day 9 in Figure 19. Statistical significance was determined between groups using Scheffe's F-Test at the $p < 0.01$ level. Values that are significantly different are denoted by (*). NS indicates no statistical significance.

Figure 18

The proliferation of bone-derived cells in response to combinations of growth factors. Bone-derived cells were plated at subconfluence in 10% FBS and incubated overnight. Cells were then incubated in 10% FBS, ■ ; 2% PPP, ◆ ; or 2% PPP supplemented with: PDGF alone (2.5 ng/ml), ▲ ; EGF (2.5 ng/ml) and TGF-B (7.0 ng/ml), × ; PDGF (2.5 ng/ml) and EGF (2.5 ng/ml), + ; PDGF (2.5 ng/ml) and TGF-B (7.0 ng/ml), ◇ ; TGF-B (7.0 ng/ml), PDGF (2.5 ng/ml) and EGF (2.5 ng/ml), □ .

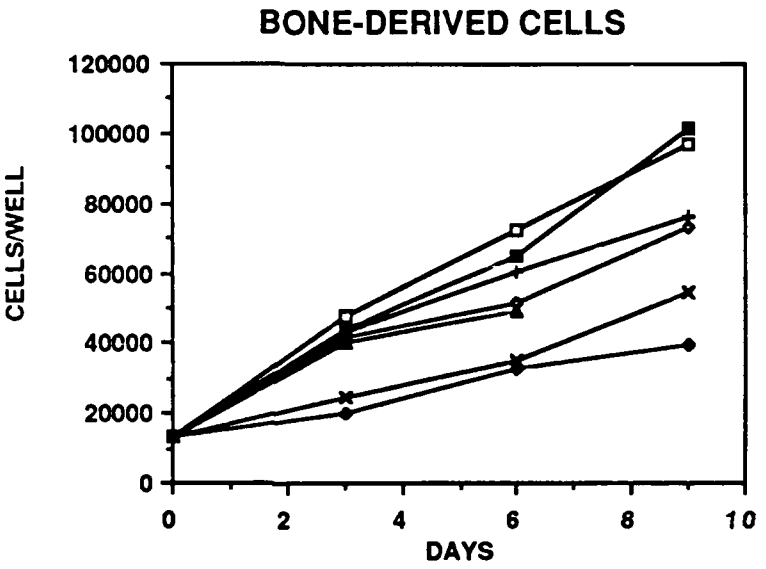
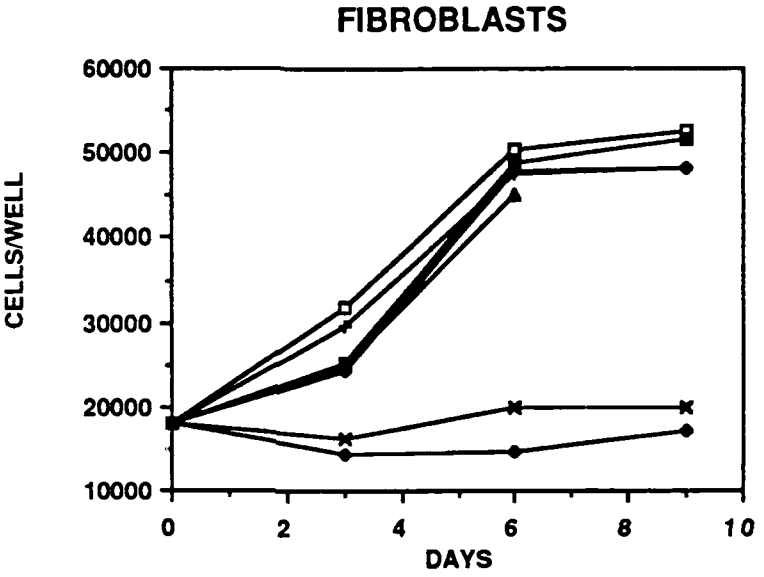

BONE-DERIVED CELLS
CELLS/WELL
120000
100000
80000
60000
40000
20000
0
0
2
4
6
8
10
DAYS

Figure 19

The proliferation of fibroblasts in response to combinations of growth factors. Fibroblasts were plated at subconfluence in 10% FBS and incubated overnight. Cells then were incubated in 10% FBS, ■ ; 2% PPP, ◆ ; or 2% PPP supplemented with: PDGF alone (2.5 ng/ml), ▲ ; EGF (2.5 ng/ml) and TGF-B (7.0 ng/ml), × ; PDGF (2.5 ng/ml) and EGF (2.5 ng/ml), + ; PDGF (2.5 ng/ml) and TGF-B (7.0 ng/ml), ◇ ; TGF-B (7.0 ng/ml), PDGF (2.5 ng/ml) and EGF (2.5 ng/ml), □ .


FIBROBLASTS
CELLS/WELL
60000
50000
40000
30000
20000
10000
0
2
4
6
8
10
DAYS

consistent with the concentrations that may be achieved in clotted serum in vivo (Carpenter and Cohen, 1979; O'Connor-McCourt et al., 1987). It has also been shown that the concentration of PDGF in clotted blood is in the range of 15-50 ng/ml, while in plasma the concentration is less than 1 ng/ml (Cochran et al., 1983; Huang et al., 1983). In the experiments described in figures 18 & 19 and tables 7 & 8 growth factors were added to DMEM supplemented with platelet-poor plasma (2%). EGF and TGF-B were utilized in multiple combinations to examine their ability to supplement and enhance the response of bone-derived cells and normal human fibroblasts to PDGF. The least stimulatory growth factor combination was EGF (2.5 ng/ml) and TGF-B (7.0 ng/ml). This growth factor combination induced only a slight increase above the negative 2% platelet-poor plasma control, which was not statistically significant. The combination of PDGF (2.5 ng/ml) and either EGF or TGF-B stimulated an intermediate level of cellular proliferation of bone-derived cells, which was similar to the results obtained with PDGF alone. Thus, this intermediate increase can be attributed primarily to the effect of PDGF and though greater than the negative control it was significantly less than 10% FBS. Only when PDGF, EGF and TGF-B were added together did the bone-derived cell population demonstrate a response equal to 10% FBS (Table 7, Figure 18). For the normal human fibroblast population, any combination of growth factors which included PDGF (2.5 ng/ml) resulted in a statistically significant increase ($p<0.01$) in cellular proliferation equal to that seen in 10% FBS. When EGF (2.5 ng/ml) and TGF-B were incubated in the absence of PDGF no increase in cellular proliferation above the 2% platelet-poor plasma negative control was observed (Table 8, Figure 19).

Additional studies were then carried out to measure the response of bone-derived cells to growth factors under defined conditions (Table 9). In these experiments growth factors were added to DMEM supplemented with 0.1% crystalline bovine serum albumin (BSA) in the absence of plasma or serum factors. The results were similar to those obtained for cellular proliferation. No single growth factor maximally stimulated DNA synthesis in the bone-derived cells, with TGF-B, IGF and EGF all slightly stimulating DNA synthesis. Of the growth factors tested, PDGF induced the largest increase. The addition of either EGF (8ng/ml) or IGF (20 ng/ml) to PDGF (8 ng/ml) did not further increase DNA synthesis in bone-derived cells. The combination of all three (EGF (8 ng/ml), TGF-B (8 ng/ml) and PDGF (8 ng/ml)) stimulated greater than a 413% increase in DNA synthesis, which was statistically equivalent to the level induced by 10% FBS. DNA synthesis equal to the 10% FBS positive control was also achieved when cells were exposed to PDGF, IGF, EGF, and TGF-B simultaneously.

Table 9

MITOGENIC RESPONSE OF BONE-DERIVED CELLS
TO GROWTH FACTORS UNDER DEFINED CONDITIONS

Treatment	CPM ± S.E.M	Percent Increase	Significance Relative To: 0.1% BSA	10% FBS
0.1%BSA	757±127	--	NS	*
EGF	916±131	21	NS	*
IGF	1103±147	46	NS	*
TGF-B	1374±104	82	NS	*
PDGF	3233±484	327	*	*
PDGF + IGF	3031±139	300	*	*
PDGF + EGF	2752±100	263	*	*
PDGF + EGF + TGF-B	3886±304	413	*	NS
PDGF + EGF + TGF-B + IGF	4845±458	540	*	NS
10%FBS	5035±156	565	*	NS

Bone-derived cells were plated at 10,000 cells/well and tested at confluence. Cells were depleted of serum factors for 48 hours in DMEM and 1% platelet-poor plasma and then incubated in DMEM containing 0.1% BSA with and without the addition of growth factors for 24 hrs. The concentrations of growth factors were as follows; IGF-1, 10 ng/ml; PDGF, 8 ng/ml; EGF, 10 ng/ml; TGF-B, 8 ng/ml. DNA synthesis was measured by ^{3}H Thymidine incorporation. Each value represents the mean of triplicate samples ± the standard error of the mean. Statistical significance was determined using Scheffe's F-test at the $p < 0.01$ level. Values that are significantly different are denoted by (*). NS indicates no statistical significance

V. DISCUSSION AND SUMMARY

Investigation into regeneration of a functional periodontal attachment involves the study of the cell types which are important in the regenerative process. Osteoblasts and gingival fibroblasts are two cell types which are felt to be important in establishing the final periodontal architecture. Current investigations indicate that when cells derived from alveolar bone and gingival connective tissue are allowed to repopulate the wound area ankylosis and resorption of the root occurs (Loe and Waerhaug, 1961; Polson and Zander, 1974; Karring et al., 1980; Lopez and Belvederessi, 1983; Isdor et al., 1986). Root cementum, an avascular, mineralized, bone-like material, is felt to function as a medium for attachment of Sharpey's fibers which bind the tooth to the surrounding bone (Armitage, 1976). Cementum is felt to be necessary for regeneration of a functional attachment apparatus, though several studies indicate that attachment of collagen fibers directly to dentin is possible. (Cole et al., 1980; Frank et al., 1983; Beertsen et al., 1985; Hanes et al., 1985; Polson and Frederick, 1985). Significantly, the periodontal ligament has been shown to possess all of the cell types felt to be necessary for periodontal regeneration (Melcher 1980; Berkovitz and Shore, 1982) with increased cell density within the periodontal ligament seen adjacent to cementum, connective tissue and alveolar bone (McCulloch and Melcher, 1983a). In addition, an undifferentiated progenitor cell population has been identified perivascularly (Gould, 1983; McCulloch, 1985) which is capable of self-renewal (Gould et al., 1980) and migration to bone and cementum where the cells presumably differentiate into osteoblasts and cementoblasts (McCulloch and Melcher, 1983b). This correlates with recent clinical

evidence which seems to indicate that coronal growth of the periodontal ligament is necessary for regeneration of the attachment apparatus (Boyko et al., 1981; Nyman et al., 1982; Gottlow et al., 1984; Isidor et al., 1986).

The studies presented here describe an initial characterization of cell types derived from human periodontia, utilizing cell morphology and biochemical characteristics. Subconfluent cells did not have distinct morphologies that were useful in distinguishing them by phase contrast microscopy. Considerable differences were noted in confluent cells. Confluent gingiva-derived cells (GF2) maintained a monolayer culture with cells oriented along parallel lines typical of fibroblasts. Confluent bone-derived cells (BP1) tended to form multi-layered colonies with randomly oriented cells. Two patterns were observed in cells derived from periodontal ligament/cementum. PL7 cells grew in a monolayer of parallel cells, in a pattern similar to GF2 cells. PL4 cells assumed a colony appearance similar to BP1 cells, growing to high density in multiple layers of randomly oriented cells.

The morphologic and colony characteristics of confluent bone-derived cells and fibroblasts have been described in the literature and are consistent with the two basic patterns reported here. Confluent adult human fibroblasts grow in uniform monolayers with adjacent cells having the same orientation (Williams et al., 1980). Bone-derived cells form dense, multilayer cell cultures (Williams et al., 1980; Nijweide et al., 1982; Wergehal and Baylink, 1984; Robey and Termine, 1985) lacking an apparent colony organization. Thus, explants GF2 and PL7 have the in vitro

appearance of fibroblasts while PL4 and BP1 cell cultures appear similar to previously described bone-derived cells.

In order to better distinguish the cell phenotypes, biochemical criteria were used. These criteria were i) PTH induced increase in c-AMP, ii) endogenous alkaline phosphatase activity, iii) PTH-induced decrease in alkaline phosphatase activity, and iv) 1,25 dihydroxyvitamin D3 induced increase in alkaline phosphatase activity. Previous reports indicate that PTH stimulates relatively large increases in c-AMP production in osteoblast cultures (Peck et al., 1973; Smith and Johnston, 1975; Wong and Cohn, 1975; Williams et al., 1980; Auf'mkolk et al., 1985). PTH has been shown to occasionally induce adenyl cyclase activity in fibroblast cultures, but the increases are small (Nijweide et al., 1981; Beresford et al., 1984; Auf'mkolk et al., 1985). Bone-derived cells have been shown to have high levels of endogenous alkaline phosphatase activity compared to fibroblasts and usually exhibit decreased alkaline phosphatase activity in response to PTH (Binderman et al., 1974; Nijweide et al., 1981; Lilja et al., 1984; Wergedal and Baylink, 1984; Whitson et al., 1984; Robey and Termine, 1985). In addition, bone-derived cell populations have been found to demonstrate a significant increase in alkaline phosphatase levels following exposure to 1,25 dihydroxyvitamin D3 (Manolagas et al., 1981; Beresford et al., 1986).

In studies presented here, cells derived from bone (BP1) exhibited biochemical characteristics consistent with an osteoblast-like phenotype; with a 6.4-fold increase in PTH-stimulated c-AMP and high levels of alkaline phosphatase which were decreased by a 15 minute and a 48 hour exposure to PTH. In addition, alkaline phosphatase was significantly increased following a 48 hour exposure to 1,25 dihydroxyvitamin D3. In

contrast, GF2 cells, derived from gingiva, had no PTH modulation of alkaline phosphatase or c-AMP and very low basal levels of alkaline phosphatase activity.

Comparison of cell cultures established from periodontal ligament tissue revealed interesting differences. Cell type PL7 was similar to GF2 cells with low levels of alkaline phosphatase activity and no PTH modulation of c-AMP or alkaline phosphatase activity. PL4 cells exhibited a different pattern. PTH induced a 19-fold increase in c-AMP, which was greater than the stimulation observed in the BP1 bone-derived cell population. An intermediate basal basal level of alkaline phosphatase was found which was significantly increased following a 48 hour, but not a 15 minute, exposure to PTH, and significantly decreased following a 48 hour exposure to 1,25 dihydroxyvitamin D3.

These results suggest a tentative classification for each cell population derived from human periodontium. Cell population GF2, obtained from dense human gingival connective tissue, exhibits the morphologic and biochemical characteristics of fibroblasts. Cell population BP1, obtained from human alveolar bone, has biochemical and morphologic characteristics similar to previously reported bone-derived cell populations. Cell population PL7, derived from human periodontal ligament/cementum tissue, was similar in all respects to GF2 cells, and thus, has a fibroblast-like phenotype. The other cell population, PL4, derived from human periodontal ligament/cementum, was not identical to either the fibroblast or the bone-derived cell populations. PL4 cells had morphologic and cell colony characteristics similar to bone-derived cells and had a large PTH-stimulated increase in c-AMP. However, they did not have a six to

eight fold higher basal level of alkaline phosphatase activity compared to fibroblasts (GF2) that is commonly observed in osteoblast cultures (personal communication Dr. Alex Valentin-Opran). In addition, PTH while causing a significant decrease in alkaline phosphatase following a 48 hour exposure did not significantly decrease alkaline phosphatase activity in PL4 cells following a 15 minute stimulation, although this criteria is not universally detected in bone cultures (Luben et al., 1976; Nejweide et al., 1981). Therefore, when the results of the morphologic and biochemical profile are cumulatively analyzed it becomes evident that the PL4 cell population behaves in a manner similar, but not identical to bone-derived cells. Since these periodontal ligament/cementum cells were explanted from a location immediately subjacent to the middle 1/3 of the root, the probable identity of this cell population has limited choices. One possibility is that these cells are cementoblasts. The culture technique utilized specifically removed cementum and periodontal ligament collagen and would allow for a selective explant of this cell type. While cementoblasts are known components of periodontal ligament tissue, the in vitro behavior of cementoblasts is not known. It is logical that cementoblasts possess a similar biochemical response to bone-derived cells since both are calcified tissues (Selvig 1965, Armitage 1976). However, cementum, unlike bone, does not serve as a calcium reservoir for the body and resorption is not induced by hormones, such as parathyroid hormone and vitamin D, which regulate calcium homeostasis under normal circumstances. This would help explain the different biochemical results which were obtained. An alternative explanation for the behavior of the PL4 cell culture is that it represents a heterogeneous population of mature

bone-derived cells and another cell type, such as immature mesenchymal cells, yielding a population of cells capable of a vigorous c-AMP response to PTH, but also having low endogenous levels of alkaline phosphatase activity. Finally, the PL4 periodontal ligament/cementum cell population may represent a mixed cell population of osteoblasts which demonstrates a low basal level of alkaline phosphatase. This should be considered possible based on the cellular morphology along with the cyclic-AMP response to PTH and the 48 hour alkaline phosphatase response to PTH and 1,25 dihydroxyvitamin D3.

While any classification of the PL4 periodontal ligament/cementum cell population is speculative, it can be concluded that these results definitely demonstrate a cell population derived from human periodontal ligament/cementum which exhibits a biochemical profile and a morphologic characterization different from fibroblasts. Recently, Sommerman and colleagues (1988) presented an initial report on a population of cells derived from the periodontal ligament which also possess an elevated level of basal alkaline phosphatase, which is in agreement with these results. However, to my knowlege this is the most extensive description in the literature of a periodontal ligament/cementum cell population which demonstrates a distinctly non-fibroblast phenotype. Also, these results further extend and confirm the biochemical response of normal human bone-derived cells to hormonal regulation as a means of differentiating these cell populations from other cells, such as fibroblasts.

Following a partial characterization of the experimental cell populations, the response of these cells to exogenous growth factors with regard to DNA synthesis and cell proliferation was examined.

Cells derived from soft tissue have been extensively studied for their response to paracrine factors (Sporn and Roberts, 1986). The results obtained in these studies have been useful in designing experiments to examine the response to growth factors in vivo (Gospodarowicz et al., 1978; Sporn et al., 1983, Grotendorst, 1984; Buckley et al., 1985; Roberts et al., 1986). In contrast, the growth factor response of bone-derived cells is less well characterized. A comparison of in vitro studies measuring the growth factor response of bone cells is complicated by significant differences in the source of the bone cells. These studies have frequently utilized immortalized bone cell lines (Graves et al. 1983; Ng et al., 1983; Graves et al., 1984; Robey et al., 1987), bone organ cultures (Canalis and Raisz, 1978; Canalis, 1981, Stracke et al., 1984; Tashjian et al., 1985; Pfeilschifter and Mundy 1987), or embryonic rat cells (Schmid et al., 1983; Hanks et al., 1986; Centrella et al., 1987). In addition, each of the above target cells has specific limitations in applying the results to adult human bone. Immortalized cell lines have been shown to have aberrant expression of oncogenes and thus, may have altered growth factor responses (Weinberg, 1985). In bone organ cultures it is difficult to assess the phenotype of the cell population and it has not been demonstrated that the response of embryonic rat bone cells is similar to adult human bone cells. Recently, non-transformed bone cells explanted from normal human bone tissue have been shown to have a stable osteoblast-like phenotype (Beresford et al., 1983a; Beresford et al., 1983b; Beresford et al., 1984; Auf'mkolk et al., 1985) and have been used to study normal bone cells in vitro. These cells have been utilized to study the response of normal human bone cells to 1,25 dihydroxyvitamin D3 (Skjodt et al., 1985;

Beresford et al., 1986), parathyroid hormone (Beresford et al., 1984; Hesch et al., 1984) and interleukin 1 (Beresford et al., 1984) as well as the synthesis and regulation of cyclic AMP (Auf'mkolk et al., 1985), osteocalcin (Beresford et al., 1984a; Beresford et al., 1984b; Auf'mkolk et al., 1985; Skjodt et al., 1985), collagen (Beresford et al., 1984b; Auf'mkolk et al., 1985), glucocorticoids (Gallagher et al., 1982; Gallagher et al., 1984), and prostaglandins (Beresford et al., 1984b; MacDonald et al., 1984). As a result of this project, the mitogenic response to growth factors of human bone cells cultured in low concentrations of platelet-poor plasma under defined conditions can now be reported. Growth factors which are thought to play an important role in wound healing were tested for their capacity to stimulate proliferation in normal human bone-derived cells having an osteoblast-like phenotype. To my knowledge this report represents the first test of the growth factor response of cells obtained from explants of normal human adult bone. These data provide insight into paracrine factors that may interact to stimulate proliferation of bone cells during osseous wound healing. In addition, the data suggest growth factors that may be useful for culturing bone cells under defined conditions.

Results presented here demonstrate that bone-derived cells proliferated in low concentrations of platelet-poor plasma. Moderate concentrations of PDGF (0.6 ng/ml) did not stimulate proliferation of bone cells, while higher concentrations of PDGF (6 ng/ml) stimulated cell proliferation but to a lesser extent than cells incubated in 10% FBS. Canalis has previously reported that high concentrations of PDGF are required to stimulate DNA synthesis in bone organ culture (Canalis, 1981).

TGF-B and EGF were mildly stimulatory although considerably less so than PDGF. Only when a combination of growth factors was tested (PDGF, TGF-B and EGF) did the proliferation of bone cells equal that of cells incubated in 10% FBS. Similar results were observed when growth factors were tested for stimulating DNA synthesis in these cells under defined conditions. Centrella and co-workers (Centrella et al., 1987) reported that TGF-B alone is highly mitogenic for cells obtained from embryonic rat calvaria. The difference between sthis study's results and Centrella and co-workers probably reflects differences in the source of bone cells and assay conditions. Interestingly, Robey, et al. (1987) have recently reported that the mitogenic affect of TGF-beta on fetal bovine cells is highly influenced by cell density. The growth factor response observed for human bone cells consistently differed from the response that this study and others have observed for fibroblasts (Antoniades and Owen, 1982; Heldin et al., 1985).

These results point out a significant difference in the growth factor requirements of bone-derived cells and fibroblasts. First, normal human fibroblasts do not proliferate in basal medium supplemented with platelet-poor plasma, while bone-derived cells do. This suggests that platelet-poor plasma contains factors which are stimulatory for bone-derived cells, but not fibroblasts. Second, concentrations of PDGF (6.0 ng/ml) which maximally stimulates DNA synthesis in other cells of mesenchymal origin stimulated the proliferation of normal human fibroblasts to the same extent as 10% FBS, while the same concentration of PDGF was less mitogenic for bone-derived cells when tested simultaneously. When PDGF was further supplemented with EGF and TGF-B, the rate of proliferation

of bone cells was equal to the rate observed for 10% FBS. This additional supplementation did not enhance the proliferation of normal human fibroblasts. There are several explanations for this difference in response between bone-derived cells and normal human fibroblasts. It is possible that the sub-optimal response of bone-derived cells to PDGF reflects a decreased sensitivity of bone cells to PDGF compared to fibroblasts. Alternatively, there could be a qualitative difference in the growth factor requirement of bone-derived cells and fibroblasts so that growth factors present in low concentrations of platelet-poor plasma enable fibroblasts to respond maximally to 6 ng/ml PDGF while they do not for bone-derived cells. This explanation is supported by evidence that supplementation of platelet-poor plasma with IGF, EGF, and TGF-B enables bone-derived cells to respond maximally to PDGF. For normal human fibroblasts this supplementation to platelet-poor plasma did not enhance the response to PDGF. Therefore, bone-derived cells may require multiple growth factors for optimal growth. A third interpretation of the above results is that the response observed for bone-derived cells reflects different growth factor requirements for different cell types within a heterogeneous cell population. It is known that in vitro bone cell populations contain a heterogeneous mixture of cells at various stages of the osteoblast lineage (Canalis, 1985). If each cell type were to have different growth factor requirements then multiple growth factors may be needed to satisfy the proliferative requirements of each cell population. If this is the case, the differential response to defined growth factors may be useful in the selection of different bone-derived sub-populations.

An interesting comparison can also be made between the results reported here and osteosarcoma cell lines. Previously it was demonstrated that the osteosarcoma cell lines U2OS (Graves et al., 1983) and MG63 (Graves et al., 1984) were able to grow in medium containing modified Eagle's medium supplemented with only platelet-poor plasma. It has been suggested that the proliferation of cells in platelet-poor plasma is indicative of the transformed phenotype (Curie, 1981). In this study normal human bone-derived cells were able to proliferate in medium supplemented with only platelet-poor plasma, while the fibroblast populations became quiescent under identical conditions. This indicates that the ability to proliferate in platelet-poor plasma depends upon the phenotype of the mesenchymal-derived cell studied and may not reflect a state of transformation.

The ability of non-transformed cells to proliferate in the above conditions may result from autocrine stimulation (Sporn and Toduro, 1980; Ozanne et al., 1982). Thus, the proliferative response to a particular growth factor may reflect the additive effect of an exogenously applied paracrine factor with endogenously produced autocrine factors. For example, fibroblasts synthesize and secrete the progression factor IGF-1, which has been shown to act as an autocrine factor in stimulating these cells (Clemmons et al., 1981; Clemmons and Van Wyk, 1985). In support of a similar process occurring in bone cells, non-transformed osteoblastic cell populations have recently been shown to produce growth factors in culture (Stracke et al., 1984; Centrella and Canalis, 1985; Farley et al., 1987; Pfeilschifter and Mundy, 1987). Thus, autocrine stimulation may be one

explanation for the ability of bone-derived cells to proliferate in platelet-poor plasma.

The examination of a population of bone cells presumed to be at various stages in the osteoblast lineage may be appropriate for studying the response to growth factors, since bone cells at various stages of differentiation may be directly or indirectly involved in osseous wound healing. In fact, Raisz and Kream (1983) have proposed that the proliferation of immature osteoblasts indirectly contributes to bone formation following bone resorption. Knowledge of the *in vitro* response of normal bone-derived cells to growth factors may be useful in devising strategies for utilizing growth factors to stimulate bone formation *in vivo* experiments. This study would suggest that a combination of growth factors, which include platelet-derived growth factor would be most stimulatory. This conclusion is not surprising since platelet-derived growth factor is released upon platelet degranulation at sites of injury, along with transforming growth factor-beta and a high molecular weight epidermal growth factor-like protein.

The previous data may also be useful in developing defined culture conditions for bone-derived cells. In order to thoroughly characterize the response of bone cells to systemic and local factors, cultures of normal human bone cells must be developed *in vitro*. *In vitro* analysis facilitates quantitation of ligand-receptor interactions, examination of co-operative or inhibitory effects of different cell modulators, investigation of secondary messages and the study of induced gene expression. Quantitation of the mitogenic effect of growth factors under defined conditions may

assist in developing culture conditions that facilitate extensive and reproducible examination of normal human bone cells.

While the response of fibroblasts and bone-derived cells to growth factors has received considerable attention in recent years, the response of periodontal ligament/cementum cells has not yet been investigated. Here the proliferation and DNA synthesis of cell populations PL-4 and PL-7 were examined with two different patterns observed. Cell population PL-4, which was shown to have morphologic and biochemical characteristics similar to the BP1, bone-derived cell population, proliferated in low concentrations of platelet-poor plasma, was not stimulated by 0.6 ng/ml of PDGF, and had increased cellular proliferation in response to 6.0 ng/ml of PDGF, which was less than that observed for 10% FBS. In contrast, PL-7 cells, which were were shown to have fibroblast-like characteristics, did not proliferate in low concentrations of platelet-poor plasma, were stimulated by 0.6 ng/ml of PDGF, and maximally stimulated by 6.0 ng/ml of PDGF to the same extent as that seen in 10% FBS. Therefore, the ability of PDGF to modulate cellular proliferation in the PL-4 and the PL-7 cell populations was found to be identical to the results obtained for bone-derived cells and the fibroblasts respectively. Since periodontal ligament cell populations respond differently to platelet-poor plasma and PDGF it may now be possible to isolate specific cell populations from the periodontal ligament. Using platelet-poor plasma, cell populations which have a bone or cementum-like phenotype could be selectively isolated, while PDGF could be utilized to selectively isolate fibroblast-like cell populations from the periodontal ligament.

Autoradiographic studies measuring the percentage of cells isolated from periodontal ligament/cementum which synthesize DNA upon PDGF stimulation also provide interesting results relative the response of fibroblasts and bone-derived cells. Both the PL-4 and the PL-7 cell populations showed a dose dependent response to PDGF when tested in medium supplemented with platelet-poor plasma or insulin and transferrin. However, for both cell types the percentage of cells responding was considerably lower than the percent of fibroblasts or bone-derived cell responding. This may indicate a high degree of cellular heterogeneity of these explants, with the majority of cells not responding even to the positive control (10% FBS), or it may indicate that a large percentage of cells are senescent. This would appear possible since cementum is a tissue which is not subject to rapid turnover and would not be expected to respond to the same extent as cells from bone or connective tissue which are responsible for continuous hard and soft tissue remodeling.

In conclusion, these results provide initial information into the response of human periodontal ligament/cementum cells to naturally occuring growth factors while further confirming the non-fibroblast phenotype of the PL-4 cell population. Also, because different cell populations from the periodontal ligament have been shown to respond differently to exogenous growth factors, the mechanism now exists to specifically isolate and culture bone or cementum-like cells from the periodontal ligament utilizing the different growth characteristics. With well characterized cell populations from the periodontal ligament along with alveolar bone and gingival connective tissue the situation now exists to investigate the factors which locally regulate these cells, particularly the periodontal

ligament cells, *in vivo*. Therefore, in addition to their regulatory effects on bone formation *in vivo*, growth factors may ultimately be used singly or in combination to allow the clinician to control the rate and amount of bone and periodontal ligament regenerated by selectively stimulating the necessary cells while simultaneously inhibiting competing cells. In this way periodontal regeneration may yet become a predictable clinical event. These experiments are an initial attempt to provide information in this area of study.

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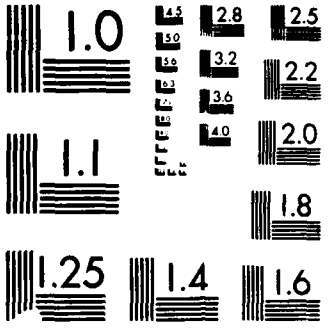

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